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**CHARACTERIZING THE ROLE OF CDK2AP1 IN PRIMARY HUMAN  
FIBROBLASTS AND HUMAN EMBRYONIC STEM CELLS**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of  
philosophy at Virginia Commonwealth University

by

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## **List of Abbreviations**

AFP	Alpha-Feto Protein
AP	Alkaline Phosphatase
BAX	BCL2-Associated Protein X
BrdU	Bromo-deoxyuridine
CDK2	Cycling Dependent Kinase 2
CDK2AP1	Cyclin Dependent Kinase 2-Associated Protein 1
ChIP	Chromatin Immunoprecipitation Assay
EB	Embryoid Body
EDTA	Ethylene-Diamine Tetra Acetic acid
HDFs	Human Dermal Fibroblasts
HEK293	Human Embryonic Kidney 293 cells
hESCs	Human Embryonic Stem Cells
hiPSCs	Human Induced Pluripotent Stem Cells
ICC	Immunocytochemistry
ICM	Inner Cell Mass
iMEFs	Inactivated Mouse Embryonic Fibroblasts
MDM2	Murine Double Minute 2
mESCs	Mouse Embryonic Stem Cells
miRNA	Micro-RNA
OCT4	Octamer-binding Transcription factor 4
OKSM	OCT4, KLF4, SOX2 and c-MYC
ONLS	OCT4, NANOG, LIN28 and SOX2
PD	Parkinson's Disease

PUMA	P53-Upregulated Modulator of Apoptosis
qPCR	Quantitative Polymerase Chain Reaction
SA- $\beta$ -GAL	$\beta$ -Galactosidase
SCNT	Somatic Cell Nuclear Transfer
Sc-shRNA	Scrambled short hair pin RNA
shRNA	Short Hairpin RNA
Tp53	Tumor Protein 53

## **Abstract**

# **CHARACTERIZING THE ROLE OF CDK2AP1 IN PRIMARY HUMAN FIBROBLASTS AND HUMAN EMBRYONIC STEM CELLS**

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Cyclin Dependent Kinase-2 Associated Protein-1 (CDK2AP1) plays an important role in cell cycle regulation, by inhibiting CDK2 and by targeting it for proteolysis. It is also known to bind the DNA polymerase alpha-primase complex and regulate the initiation step of DNA synthesis. Its overexpression has been shown to inhibit growth, reduce invasion and increase apoptosis in a number of cancer cell lines. In studies in which mouse embryonic stem cells (mESCs) with targeted deletion of the *Cdk2ap1* gene were used, Cdk2ap1 was shown to be required for epigenetic silencing of Oct4 during differentiation. The goal of this thesis was to examine the role of CDK2AP1 in somatic cells (primary human dermal fibroblasts (HDFs)) and human embryonic stem cells (hESCs) and specifically assess its impact on proliferation, self-renewal and differentiation.

In the first part of this study, using a short-hairpin RNA (shRNA) approach, we investigated the effect of CDK2AP1 downregulation in HDFs. Outcomes indicated: (a) reduced proliferation, (b) premature senescence, (c) cell cycle alterations, (d) DNA damage, and (e) an increase in p53, p21, and the p53-responsive apoptotic genes *BAX* and *PUMA*. Simultaneous downregulation of p53 and CDK2AP1 in HDFs confirmed that observed phenotype was p53 dependent.

In the second part of this study, using a shRNA approach, we investigated the role of CDK2AP1 on hESC fate associated with self-renewal and differentiation. We found that CDK2AP1 knockdown in hESCs resulted in: (a) reduced self-renewal (b) enhanced differentiation (c) cell cycle alterations and (d) increase in p53 expression. Results indicate that the knockdown of CDK2AP1 in hESCs enhances differentiation and favors it over a self-renewal fate.

Thus, this study has successfully identified novel functions for CDK2AP1, as its knockdown has a significant impact on self-renewal, differentiation and senescence. Results obtained from this study could contribute to development of directed differentiation strategies for generating uniform populations of differentiated phenotypes from hESCs for clinical applications.

## **CHAPTER 1**

### **Introduction and Review of the Literature**

#### **1.1. Human Embryonic Stem Cells**

Embryonic stem cells, the most studied type of pluripotent stem cells, are derived from the inner cell mass (ICM) of the preimplantation blastocyst (Smith 2001). The derivation process involves the seeding of these cells onto mitotically inactivated mouse embryonic fibroblasts (MEF), followed by the expansion of the outgrowth into established human embryonic stem cell (hESC) lines. The two main defining characteristics of hESCs are their unlimited capability to symmetrically divide and self-renew, and their ability to differentiate into numerous cells derived from any of the three germ layers (i.e., ectoderm, mesoderm and endoderm).

The first successful derivation of hESCs was reported by Thomson and colleagues, wherein hESC lines were established by isolating cells from the ICM and plating them onto mitotically inactivated MEFs (Thomson, Itskovitz-Eldor et al. 1998). Subsequently, Reubinoff and colleagues were able to establish two diploid hESC lines and propagate them in culture for extended periods while maintaining the expression of hESC markers. In addition, the group was



able to demonstrate the differentiation potential of hESCs under in vitro conditions. They specifically showed that neural progenitors could be isolated from differentiating hESCs and could be further induced to differentiate and form mature neurons (Reubinoff, Pera et al. 2000). This study provided evidence that hESCs could be utilized as a model to study early human embryonic development and also serve as a useful source of cells for applications in transplantation therapies.

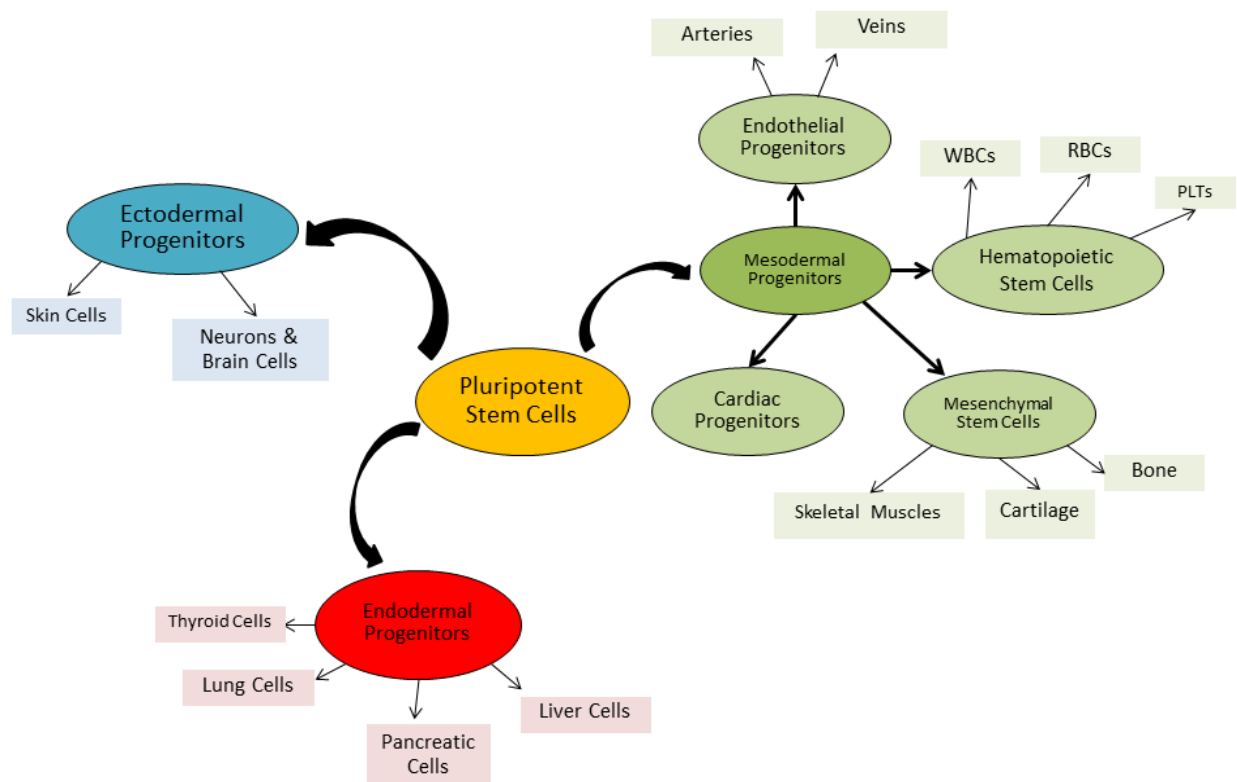
The use of hESCs as a source for cell therapeutic approaches (Figure 1) is currently being examined for a number of diseases. For instance, one of the earliest studies to document the potential for hESCs in treating myocardium diseases demonstrated a great ability of these cells to regenerate a damaged heart muscle (Laflamme, Chen et al. 2007). In this study, hESCs were successfully differentiated to form cardiomyocytes using a process scaled for clinical production and showed normal contractile function along with appropriate responses to cardiac drugs (Laflamme, Chen et al. 2007). These cells were then transplanted into animal models of myocardial infarction, wherein the cells engrafted and improved the left ventricular function (Laflamme, Chen et al. 2007).

Another promising therapeutic approach that has been explored is the transplantation of pulmonary progenitors cells derived from hESCs to regenerate endogenous lung cells damaged by injury or disease. In a recent study, researchers utilized a nude mouse model of acute lung injury to examine the therapeutic potential of alveolar type II epithelial cells which were derived from hESCs (Spitalieri, Quitadamo et al. 2012). HESCs were successfully differentiated in culture to alveolar type II pneumocytes, after which the ability of the differentiated cells to induce recovery in vivo was examined. Following injection of the committed cells into the

mouse models, significant recovery of fibrosis and inflammation was observed when compared to non-injected mice. The recovery was demonstrated by restoration of the lung functionality measured by blood oxygen saturated levels (Spitalieri, Quitadamo et al. 2012).

Neurodegenerative diseases are another important target for stem cell-based therapy. Parkinson's disease (PD), which is characterized by the progressive loss of dopaminergic neurons, is one that is being initially targeted in the context of stem cell-based therapies. The focus in this approach is the functional recovery by replacement of only one cell type (dopaminergic neurons), which thus enables the relative ease in testing the true potential of hESCs in regenerative medicine (Iyer, Alsayegh et al. 2009). A number of studies that involved the transplantation of dopaminergic neurons into the striatum of PD animal models have shown a reduction in PD-related motor deficits, increased release of dopamine, and synaptic integration of dopaminergic neurons (Perrier, Tabar et al. 2004, Takagi, Takahashi et al. 2005, Yang, Zhang et al. 2008). Such studies provided strong evidence towards the potential of hESC-derived dopaminergic neuron transplantation as a possible treatment option for PD.

Although hESCs have great potential, there are few hurdles that need to be solved before they can be used in the clinic. In addition to the ethical issues associated with hESC research which also initially resulted in restriction of federal funding, safety remains the main challenge. It is critical that transplants derived from hESCs contain, as much as possible and almost completely, only enriched populations of differentiated cells. The existence of a very small number of pluripotent cells thus has the potential to increase the risk for teratoma formation



**Figure 1: Potential of Pluripotent Stem Cells.** Embryonic stem cells derived from the inner cell mass of blastocyst stage embryo differentiate to almost any desired cell type, highlighting their importance and potential in regenerative medicine.

(Li, Christophersen et al. 2008). Therefore, improved methods are needed to direct differentiation into the desired cell types and to minimize the presence of undifferentiated cells.

Another major issue with the safety of hESC-based cell therapy is the issue of allogeneic graft rejection given that the original cell source is not patient-specific. One proposed solution has been to generate a large hESC bank from which histocompatible matches could be selected. However, even with a close match, transplant recipients may need to use immunosuppressive drugs for the rest of their lives, which adds additional risks (Nakajima, Tokunaga et al. 2007, Taylor, Bolton et al. 2005).

## **1. 2. Cell Cycle Regulation in ESCs**

ESCs are known to have a cell cycle regulation network that is substantially different from somatic cells, with cycling time known to be significantly faster when compared with terminally differentiated cells. For example, mESCs progress through a single cell cycle in about 8-11 hours (White, Dalton 2005), while hESCs progress through the cell cycle in 15-16 hours (Becker, Ghule et al. 2006), while many somatic cells progress through the cell cycle in ~24 hours in cell culture (Fluckiger, Marcy et al. 2006).

The short cell-cycle duration for ESCs results, primarily, from a very short G1 phase that lacks a restriction point and a G1 checkpoint. While somatic cells require exogenous growth factors to enter the cell cycle, ESCs do not. In addition, in contrast to somatic cells, ESCs are not subject to contact inhibition and do not enter quiescence upon withdrawal of growth factors (Savatier, Lapillonne et al. 1996, Schratt, Weinhold et al. 2001) . This independence from growth factors is most likely caused by the hyperphosphorylation of the retinoblastoma protein (pRb)

that in turn, causes continuous activation of the E2F target genes resulting in faster progression of the cells into the S phase. Indeed, it was observed that ESCs spend a very short time in the G1 phase (2 hours in the mouse ESCs) of the cell cycle (Koledova, Kramer et al. 2010).

In mESCs, all cyclins are expressed at comparable levels throughout the cell cycle, and exhibit higher expression when compared with somatic cells (Fujii-Yamamoto, Kim et al. 2005). In addition, mESCs do not express CDK inhibitory proteins of the Ink4 and Cip/Kip families (Savatier, Lapillonne et al. 1996, Faast, White et al. 2004, Savatier, Huang et al. 1994). For this reason, most CDKs are active throughout the cell cycle with the exception of CDK1-Cyclin B complex which only becomes active just before the onset of mitosis (Stead, White et al. 2002) and CDK4-Cyclin D1 which shows no activity in mESCs (Faast, White et al. 2004). The driving force underlying the rapid cell cycle progression in mESCs is the high activity of Cdk2. Chemical inhibition of Cdk2 activity prolongs cell-cycle duration and slows the progression of mESCs through the G1 phase of the cell cycle. Overexpression of the CDK2 inhibitor p21, for example causes similar effects.

In hESCs, the cell cycle regulation and profile is not overtly different from mESCs, with the exception that hESC cell cycle regulators show cell cycle phase-dependent expression (Neganova, Zhang et al. 2009). Similar to mESCs, hESCs also exhibit a short G1 phase (Becker, Ghule et al. 2006) which is most likely caused by high mRNA levels of CDK4-Cyclin D2 and low levels of p21, p27, and p57 (Becker, Stein et al. 2007, Sengupta, Nie et al. 2009). Similar to mESCs, members of the Ink4 family are expressed at very low levels in hESCs (Miura, Luo et al. 2004), and thus contributes to high activity of the CDKs. In addition, CDK2 was shown to display the overall highest kinase activity in hESCs (Neganova, Zhang et al. 2009). Also similar

to mESCs, any type of inhibition of CDK2 activity delays the transition from G1 phase to S phase of the cell cycle with directed knockdown of CDK2 leading to G1 arrest in hESCs (Neganova, Zhang et al. 2009, Filipczyk, Laslett et al. 2007). Taken together, these data indicate a crucial role for CDK2 for G1 phase regulation in both mESCs and hESCs.

### **1.3. Cell Cycle Regulation in Somatic Cells**

In response to growth factors and/or mitogenic signals, Cyclin D-CDK4/CDK6 complex is activated and promotes the phosphorylation of the pRb protein, leading to the progression of cells through the G1 phase of the cell cycle (Resnitzky, Reed 1995). E2F dependent induction of Cyclin E and CDC25A results in further phosphorylation of the pRb protein and the transition from G1 to S phase of the cell cycle. This is mainly controlled by the kinase activity of the Cyclin E/CDK2. The Cyclin A/CDK2 complex then controls the progression through S and G2 phases of the cell cycle. Lastly, Cyclin A and B/CDK1 complexes regulate entry into mitosis and control early mitotic events. Somatic cells have a longer doubling time (around 24 hrs) when compared to ESCs (Koledova, Kramer et al. 2010) and generally have lower levels of cyclins when compared to ESCs. (Fujii-Yamamoto, Kim et al. 2005). In addition, tumor suppressors like p21, p27 and p57 are expressed in higher levels in somatic cells than in ESCs (Koledova, Kramer et al. 2010).

### **1.4. The Relationship Between Cell Cycle and Cell Fate Decisions**

The observed changes in cell cycle regulation and expression of cell cycle regulatory proteins during the onset of differentiation in stem cells, indicates a strong link between cell cycle regulation and the loss of pluripotency. Additional evidence has also come from studies

that examined adult stem cells and the switch they go through when they divide symmetrically or asymmetrically. A study that examined the differences in cell cycle between neural progenitors that are self-renewing (dividing symmetrically) and neural progenitors that undergo neuron-generating divisions (asymmetric divisions), revealed that the former cell group have a significantly shorter cell cycle (Calegari, Haubensak et al. 2005). Furthermore, the group also observed that cells dividing at the basal side of the ventricular zone have a longer G2 phase than the self-renewing cells dividing at the ventricular surface (Calegari, Haubensak et al. 2005). Interestingly, in another study it was shown that inhibition of CDKs that lengthen the G1 phase (but do not arrest in G1) of the cell cycle in whole mouse embryo culture, induced premature neurogenesis in those embryos (Calegari, Huttner 2003). It was also shown that overexpression of the CDK4-Cyclin D1 complex in neural stem cells resulted in the shortening of the G1 phase and was sufficient to promote the switch from neurogenesis to the generation and expansion of basal progenitors (Lange, Huttner et al. 2009).

Studies in mouse and hESCs have also shown that the connection between cell cycle, self-renewal and pluripotency exists in pluripotent stem cells. The general phenomenon is that prolongation of any of the cell cycle phases and the inhibition of cell cycle progression results in the differentiation of ESCs. An important finding was that the downregulation of CDK2 using a chemical inhibitor in hESCs resulted in a delay in the G1-S progression (Filipczyk, Laslett et al. 2007) while a shRNA mediated knockdown of CDK2 in hESCs resulted in the abrogation of G1-S phase progression with cells arrested in the G1 phase of the cell cycle (Neganova, Zhang et al. 2009). In both cases (chemical inhibition and knockdown), differentiation of hESCs was induced and measured by reduced expression of OCT4 (Neganova, Zhang et al. 2009, Filipczyk, Laslett et al. 2007).

The above mentioned studies suggest that CDK2 plays a crucial role in the self-renewal and differentiation of mouse and hESCs. However, knockout studies that were performed in mice, showed that CDK2 is not essential as homozygous *Cdk2* knockout mice develop normally in all aspects, except in meiosis. These knockout mice demonstrated that Cdk2, although not essential for the embryos to develop is however indispensable for meiosis. A more likely explanation is that other cell cycle regulation proteins could be compensating for the absence of Cdk2. In addition, the state of ESCs in the blastocyst only represents a transient state in early development or embryogenesis, fated to differentiate and be under the influence of multiple signals that regulate their fate. For that reason, the absence of CDK2 might not affect the cell fate decisions in the ESCs of the developing embryo (Koledova, Kramer et al. 2010).

It is well acknowledged that prolongation of cell cycle in ESCs will most likely lead to the differentiation of the cells. Prolongation of the cell cycle due to genotoxic stress in ESCs, was shown to result in differentiation as opposed to senescence or apoptosis and interpreted as an attempt of ESCs to keep the stem cell pool free of any genetic instability (Lin, Chao et al. 2005). This checkpoint that regulates the switch between maintaining self-renewal versus undergoing differentiation due to genotoxicity or DNA damage, was attributed to a number of proteins, including Ataxia Teleangiectasia Mutated (ATM) and p53 (Inomata, Aoto et al. 2009, Lin, Chao et al. 2005). However, it is still not fully clear how exactly self-renewal regulation and DNA damage responses are linked with a high possibility that this link might differ from one stem cell type to another. A perfect example is that when DNA damage was induced in mESCs, p53 was activated leading to the differentiation of the cells by repression of the pluripotency marker Nanog by direct binding of p53 to its promoter (Lin, Chao et al. 2005). However, when p53 was activated in hESCs by treating with the p53 activator, nutlin, the cells accumulated in the G0/G1



phase of the cell cycle that coincided with the elevation of the differentiation markers and a decrease in the pluripotency genes through the upregulation of the p53 target gene p21 (Maimets, Neganova et al. 2008).

The link between cell cycle control and pluripotency is strongly supported with the findings that show a link between pluripotency markers and major cell cycle regulators. In an interesting study, a group aimed to investigate the effect of NANOG overexpression in hESCs (Zhang, Neganova et al. 2009). Findings indicated that multiple hESC lines with NANOG overexpressed divided significantly faster than the control cells. To test whether this was caused by an increase in proliferation or a decrease in apoptosis, Bromodeoxyuridine (BrdU) incorporation assay was performed along with apoptosis assay, and it was found that there was no change in rate of apoptosis; however, there was a significant increase in BrdU incorporation. In addition, the group noticed the absence of the spontaneous and typical signs of differentiation in the NANOG overexpressing hESCs, which was a pattern normally exhibited in control hESC cultures.

By examining the cell cycle profile of the NANOG overexpressing hESCs, it was found that the overexpression of NANOG resulted in the acceleration of the progression from G1 to S phase of the cell cycle (Zhang, Neganova et al. 2009). Western Blot analysis to examine the levels of cell cycle regulatory proteins indicated that the levels of CDK6 and CDC25A were significantly increased with NANOG overexpression. Chromatin Immunoprecipitation (ChIP) assay revealed that NANOG regulated CDK6 and CDC25A at the transcriptional level. Knockdown studies of CDK6 and CDC25A in hESCs revealed that both proteins are important in G1-S progression, and their downregulation resulted in a delay in that progression. Furthermore, knockdown of CDC25A also resulted in a delay in the G2/M phase progression. Collectively, these data suggest

that NANOG, one of the major pluripotency markers, directly controls major cell cycle regulatory proteins, which clearly highlights the link between cell cycle regulation and pluripotency (Zhang, Neganova et al. 2009).

Additional studies have also focused on the link between cell cycle regulation and cell fate decision. For example, OCT4 and SOX2 were also shown to regulate the expression of key cell cycle regulatory proteins like, CDK1, CDC27 and cyclin D1 (Boyer, Lee et al. 2005). Additional evidence of a link between the self-renewal machinery and cell cycle regulation has been provided by a study (Chavez, Bais et al. 2009) in which researchers carried out an integrated analysis of high-throughput data (ChIP-on-chip and RNAi experiments along with promoter sequence analysis of putative target genes) and identified a core OCT4 regulatory network in hESCs. The results suggest that E2F, a major factor in the G1-S phase transition, may also function as a regulatory cofactor of OCT4 at the promoter of OCT4 target genes. In addition, the results also revealed that the Origin recognition subunit 1-like protein (*ORC1*), which is a direct target of E2F involved in DNA replication and mitosis, is also part of the core OCT4 regulatory network.

Another important aspect of the relationship between the state of pluripotency and cell cycle status, is cell cycle regulation by microRNA's (miRNAs) (Qi, Yu et al. 2009). For example, miRNA 195 and miRNA 372, which are highly expressed in hESCs, regulate two tumor suppressor genes, respectively: WEE1, which encodes a negative G2/M kinase modulator of the Cyclin B/CDK complex and CDKN1A, which encodes p21, a Cyclin E/CDK cyclin dependent kinase inhibitor that regulates the G1/S transition (Figure 2). Together, all these studies

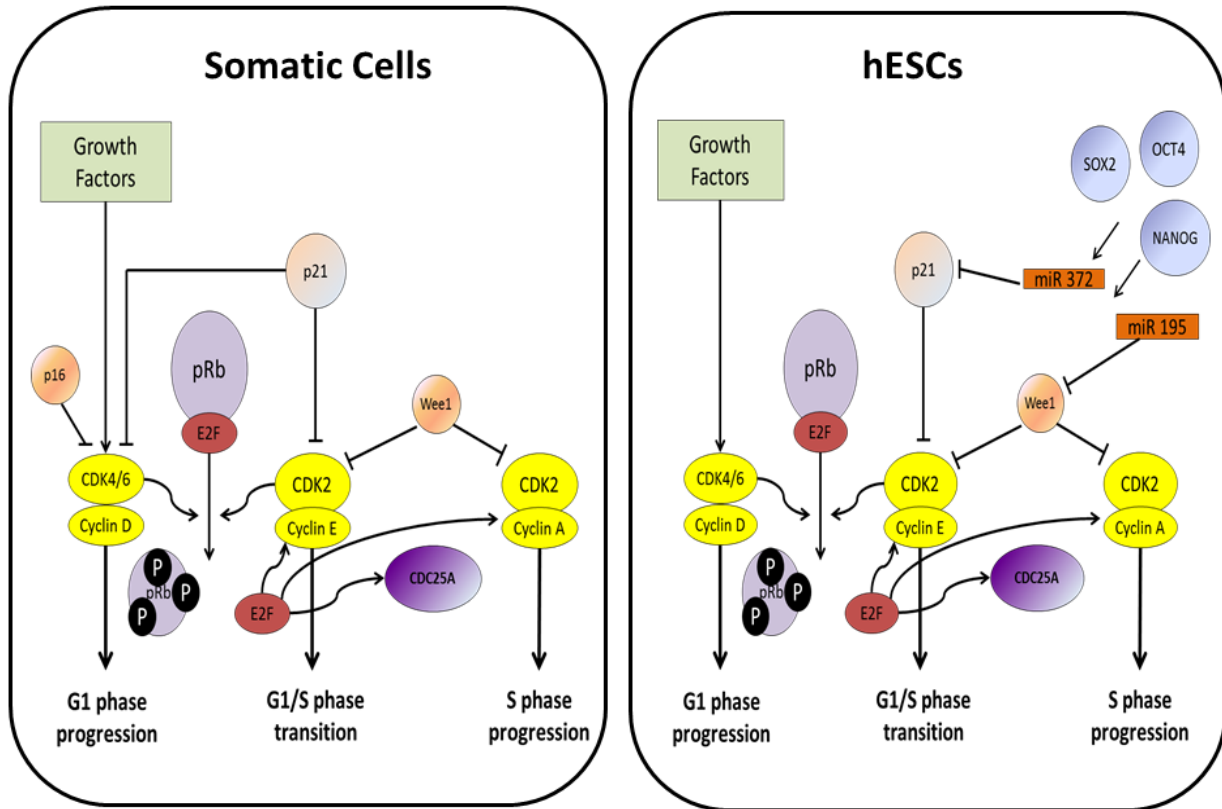
demonstrate that pluripotency dictates specific patterns in cell cycle control, that facilitate continuous self-renewal of pluripotent cells.

### **1.5. Cell Cycle Control and the Induction of Pluripotency**

Since the discovery of reprogramming and the induction of pluripotency in somatic cells, many studies have focused on modifying the standard protocol for iPSC generation in order to enhance the efficiency of the process and to eliminate the need for any integration or the use of exogenous proto-oncogenes. Given that iPSCs resemble ESCs in their cell cycle profile, it was intuitive to manipulate cell cycle regulators in somatic cells to alter their cell cycle profile and bring closer to that of ESCs, when trying to enhance iPSCs generation. One of the earliest studies (Zhao, Yin et al. 2008), utilized a shRNA to downregulate p53 in mouse fibroblasts to examine the effect of this knockdown on the efficiency of iPSC generation. Interestingly, the knockdown of p53 enhanced the efficiency of reprogramming more than a 100 fold, indicated by the increase in the number of alkaline phosphatase (AP) positive colonies.

Another study demonstrated that p53 deletion enhanced the efficiency of plasmid based reprogramming which did not require the integration of exogenous factors in the host cell genome (Hong, Takahashi et al. 2009). In the same study, it was revealed that the pathway responsible for this enhancement in reprogramming efficiency upon the deletion of p53, is the p53-p21 pathway. It was shown that p21 was downregulated in p53 null fibroblasts and resulted in the observed improvement in efficiency. Furthermore, it was also suggested that p53 downregulation may be enhancing reprogramming by promoting immortalization (Mali, Ye et al. 2008).

Another study that also supports that hypothesis revealed that by knocking down the components of the Ink4a/Arf locus (also known as Cdkn2a locus) which encodes the CDK inhibitors p16 nk4a and p15 Ink4b and the positive regulator of p53, p19 Arf, reprogramming efficiency was significantly increased (Utikal, Polo et al. 2009). In addition, colonies were generated with up to three-fold faster kinetics. Interestingly, the reprogramming factor Klf4, was also shown to function by suppressing the levels of p53, thus demonstrating its role in enhancing efficiency of iPSC generation (Rowland, Bernards et al. 2005). Furthermore, the reprogramming factor Lin28, which is known to positively regulate Cyclins A and B and CDK4 (Xu, Zhang et al. 2009), was shown to enhance reprogramming efficiency by increasing proliferation (Hanna, Saha et al. 2009). Additional evidence of the link between cell cycle regulation and reprogramming has been provided by a study that demonstrated an increase in reprogramming efficiency by using Rem2 GTPase through two different means. In addition to its effect in repressing p53, Rem GTPase also helps increasing the expression and the nuclear localization of Cyclin D1, which results in increased progression through cell cycle (Edel, Menchon et al. 2010). Taken together, all these studies strongly suggest that cell cycle control is a rate-limiting step in the process of reprogramming and that reprogramming cannot occur independently without a significant change in the cell cycle profile of the starting cell population.



**Figure 2: Regulation of G1 Phase Progression in Somatic Cells and hESCs.** Following mitogenic stimulation, Cyclin D1 is synthesized and it stimulates CDK4/6 and together they form a complex. Cyclin D-CDK4/6 complex then phosphorylates pRb and that releases the E2F which in turn activates proteins like Cyclin E. By forming a complex with CDK2, the Cyclin E-CDK2 complex further phosphorylates the pRb releasing more E2F to activate S phase related genes. Cyclin E-CDK2 complex also phosphorylates pre-replication complexes and is important in centrosome duplication. Human ESCs have higher levels of cyclins and lower levels of the negative regulators of the CDKs.

## **1.6. Cyclin Dependent Kinase-2 Associated Protein-1 (CDK2AP1): Linking Cell Cycle Control and cell Fate**

Human CDK2AP1 is a highly conserved, ubiquitously expressed gene located on chromosome 12q24 and is a 115-aa nuclear polypeptide (Tsuji, Duh et al. 1998), while its murine equivalent is located on chromosome number 5 and has only 3 amino acids deviations from the human CDK2AP1 (Kim, Ohyama et al. 2005). CDK2AP1 is also called Deleted in Oral Cancer-1 (Doc1) as it was found to be downregulated in about 70% of oral cancers (Shintani, Mihara et al. 2001). In vitro studies have revealed that CDK2AP1 mainly functions by regulating the S phase of the cell cycle, with studies showing that binding of CDK2AP1 to the DNA polymerase alpha /primase complex specifically prevents the initiation step of the DNA replication process (Matsuo, Shintani et al. 2000). However, it is still not clear whether this is caused by a direct inhibition of the DNA polymerase alpha/primase complex, or by the CDK2AP1 downregulation of CDK2, which is responsible for the stimulation of DNA replication by phosphorylating the DNA polymerase alpha/primase complex (Matsuo, Shintani et al. 2000).

CDK2AP1 has also been shown to mediate the growth inhibitory effects of the Transforming Growth Factor Beta1 (TGF-beta1) (Hu, Hu et al. 2004). In the study reported by Hu and colleagues, it was shown that TGF-beta1 induced CDK2AP1 expression transcriptionally, which in turn, mediated the growth inhibitory activity of TGF-beta1. The group generated HaCaT cells with CDK2AP1 antisense and showed that the reduction in CDK2AP1 levels resulted in an increase in the levels of CDK2-associated kinase activity, an increase in the levels of phosphorylated pRb and increase in proliferation. Additional studies in mouse

keratinocytes that were deficient in CDK2AP1 showed that the TGF-beta1 response was significantly diminished in those cells when compared to cells expressing normal levels of CDK2AP1.

Numerous studies have demonstrated the association of CDK2AP1 with multiple types of cancer. It has primarily been studied in oral cancers, where TGF-beta1 response was shown to be decreased. It was reported that there was a decrease in the levels of TGF-beta1 receptor II (TGF- $\beta$ RII) that coincides with the transition from carcinoma to metastasis (Meng, Xia et al. 2011), with the decrease demonstrated to exhibit a statistically significant correlation with a decrease in the expression of CDK2AP1, p21 and p27 (Peng, Shintani et al. 2006). In addition, CDK2AP1 expression was also found to be decreased in prostate cancer cells. Upon forced overexpression of CDK2AP1 in prostate cancer cells, cell cycle arrest and apoptosis were induced along with a decrease in cell invasion capacity (Zolochovska, Figueiredo 2009). Furthermore, low levels of CDK2AP1 in the gastric tissue of gastric cancer patients, correlated with lower life expectancy, while levels of p53 had no significant correlation (Choi, Sohn et al. 2009).

CDK2AP1 has lately gained importance in the field of stem cell research, with initial studies identifying it as one of the stem cell-specific genes that are enriched in both embryonic and adult stem cells (Ramalho-Santos, Yoon et al. 2002). It has also been identified as one of many genes that are expressed in early stage preimplantation embryos (Sharov, Piao et al. 2003). An extensive study analyzed the phenotypic characteristics of a homozygous *Cdk2ap1* knockout mESCs (Kim, Deshpande et al. 2009). Upon removal of the Leukemia Inhibitory Factor (LIF), which is traditionally used to maintain the symmetric division of mESCs, the wild type (*Cdk2ap1*<sup>+/+</sup>) mESCs started showing typical morphological changes that are associated with

differentiation. On the other hand, the *Cdk2ap1* knockout (*Cdk2ap1*<sup>-/-</sup>) mESCs appeared normal and did not show any signs of differentiation. In addition, although *Cdk2ap1*<sup>+/+</sup> mESCs lost their alkaline phosphatase (AP) activity, *Cdk2ap1*<sup>-/-</sup> cells were still AP positive. Interestingly, this observed phenotype was maintained for up to 20 days in culture. These results indicated that differentiation was altered in *Cdk2ap1*<sup>-/-</sup> mESCs and that there was increased maintenance of self-renewal even under differentiation stimulus, with the reversal of the observed phenotype upon introduction of exogenous *Cdk2ap1*.

Additionally, the mRNA expression of most of the stem cell-related genes were examined by quantitative reverse transcription-PCR using embryoid bodies obtained from *Cdk2ap1*<sup>+/+</sup> and *Cdk2ap1*<sup>-/-</sup> mESCs. Quantitative PCR analysis revealed that levels of *Oct4* and *Nanog* were significantly higher in embryoid bodies obtained from *Cdk2ap1*<sup>-/-</sup> mESCs when compared to *Cdk2ap1*<sup>+/+</sup> embryoid bodies. In an attempt to confirm that the findings were caused by the loss of *Cdk2ap1*, stable clones from *Cdk2ap1*<sup>-/-</sup> mESCs were generated in which *Cdk2ap1* was ectopically expressed. After differentiating the clones to embryoid bodies and assessing the levels of *Oct4* and *Nanog*, it was found that the exogenous *Cdk2ap1* caused repression in the expression of *Oct4* and *Nanog* in a way similar to *Cdk2ap1*<sup>+/+</sup> embryoid bodies. Furthermore, qPCR analysis showed a decrease of differentiation markers [Heart and Neural Crest Derivatives-expressed protein-1 (*Hand1*) and homeobox protein *Cdx2*] in *Cdk2ap1*<sup>-/-</sup> embryoid bodies compared to *Cdk2ap1*<sup>+/+</sup>, and the decrease was reversed in the *Cdk2ap1*<sup>-/-</sup> embryoid bodies that ectopically expressed *Cdk2ap1*. These findings support the possibility that *Cdk2ap1* may play an important in regulating stem cell markers during differentiation.



Based on previous reports showing that the pRb is hyperphosphorylated when *Cdk2ap1* is deleted (Shintani, Ohyama et al. 2000), and because pRb was shown to play a role in ES cell differentiation (White, Stead et al. 2005) it was intuitive to test whether the loss in differentiation capability associated with *Cdk2ap1* deletion, was a result of the hyperphosphorylation of pRb. Initially, the levels of phosphorylated pRb at serine<sup>788</sup>, which is a site specific for Cdk2 activity, were measured and were found to be significantly higher in *Cdk2ap1*<sup>-/-</sup> than wild type and heterozygote knockout mESCs. This was reversed after the addition of exogenous *Cdk2ap1* in *Cdk2ap1*<sup>-/-</sup> mESCs, and pRb became dephosphorylated. To test whether this observed hyperphosphorylation of pRb is responsible for the blockade of differentiation, multiple mutant forms of pRb were introduced to *Cdk2ap1*<sup>-/-</sup> mESCs. The findings revealed that only the S788A mutant (mutating the site of Cdk2 induced phosphorylation) could restore differentiation capability, clearly demonstrating that the phosphorylation at the serine<sup>788</sup> is responsible in part for the absence of differentiation in *Cdk2ap1*<sup>-/-</sup> mESCs (Kim, Deshpande et al. 2009).

By assessing the effect of the introduction of the pRb mutant S788A on levels of embryonic stem cell markers, it was found that Oct4 was significantly reduced independent of LIF removal, while Nanog, Sox2 and Rex1 showed more LIF dependence (Kim, Deshpande et al. 2009). Interestingly, the differentiation markers Hand1 and Brachyury, showed an increase in their levels independent of LIF treatment. On the other hand, introduction of wild type pRb did not induce any changes in the expression of pluripotency genes or differentiation markers. Collectively, these findings indicate that the loss in differentiation potential of *Cdk2ap1*<sup>-/-</sup> mESCs is mediated in part through the disruption of pRb regulation through hyperphosphorylation of serine 788. Overall, this study demonstrated the critical role of *Cdk2ap1* in mESC differentiation and provided another strong evidence of a link between cell cycle control and cell fate decisions.

## 1.7. CDK2AP1: Its Role in Epigenetics and ESC Differentiation

During early stages of embryogenesis, embryonic stem cells are subjected to an extensive array of signals, like the changes in gene expression at specific stages of embryonic development and communications between cells and their surrounding environment (Koledova, Kramer et al. 2010). An important approach for embryonic stem cells to regulate their response to all these signals is through epigenetics. Epigenetic marks, like DNA methylation, histone modifications and bivalent chromatin, allow for many genes to be turned on when the appropriate developmental time is scheduled. Epigenetic mechanisms are thus essential for embryonic stem cells to regulate gene expression without altering nucleotide sequence.

During embryogenesis, regulation of genes is mediated epigenetically by DNA methyltransferases (DNMT) and/or nucleosome remodeling complexes, such as the nucleosome remodeling and deacetylase (NuRD) complex. These can modify DNA or histones as a response to certain developmental cues, and changes the gene expression profile in an appropriate manner (Muller, Leutz 2001, Tong, Hassig et al. 1998). As ESCs differentiate, *Oct4* expression is downregulated by an increased methylation of its promoter. Alterations of the methylation process by DNMT inhibitors and/or alterations of histone deacetylation by histone deacetylation inhibitors such as Trichostatin, results in failed differentiation of ESCs, which clearly suggest a critical role of epigenetics in stem cell fate regulation (Tsuji-Takayama, Inoue et al. 2004).

Recently, Cdk2ap1 has been linked to epigenetic regulation and embryonic stem cell differentiation. Cdk2ap1 was identified to be part of the epigenetic repressor, the NuRD complex, and was shown to colocalize with the methyl binding domain-2 (Mbd2) protein subunit

of NuRD (Spruijt, Bartels et al. 2010). Gene silencing during the differentiation of mESCs is mediated by the NuRD complex and is required for the mESCs to exit pluripotency (Kaji, Caballero et al. 2006). Mouse ESCs with MBD3 (a subunit of NuRD complex) deleted, are defective in differentiation and display persistent self-renewal (Kaji, Caballero et al. 2006). Interestingly, this phenotype exactly matches the one seen in mESCs with *Cdk2ap1* removed (Deshpande, Dai et al. 2009). In this study (Deshpande, Dai et al. 2009), *Cdk2ap1* knockout mESCs were generated and their differentiation potential assessed. It was found that lower number of EBs could be generated from *Cdk2ap1*<sup>-/-</sup> mESCs compared to wild type mESCs. More interestingly, in day 8 *Cdk2ap1*<sup>-/-</sup> Ebs, there was still high expression of the pluripotency gene *Oct4*. Because *Oct4* silencing is primarily mediated epigenetically by increased methylation of its promoter, methylation-specific PCR (MS-PCR) and bisulfite sequencing was performed on day 8 Ebs from *Cdk2ap1* knockout and wild cells, which revealed a significant reduction in methylation of Oct4 promoter in *Cdk2ap1*<sup>-/-</sup> Ebs compared to wild type. The hypermethylation of Oct4 promoter following LIF removal was restored in *Cdk2ap1*<sup>-/-</sup> after the introduction of exogenous *Cdk2ap1*. Because Cdk2ap1 was reported to be part of the NuRD-MBD3 complex (Le Guezennec, Vermeulen et al. 2006) and because of the overlap in the phenotype observed when Cdk2ap1 or Mbd3 were deleted in mESCs, co-immunoprecipitation studies were performed to determine if Cdk2ap1 interacted with Mdb3. Using bacterially expressed deletion mutants of Cdk2ap1 and Mbd3, a sequence of 8 amino acids in the N-terminus of Cdk2ap1 was found to be used for the interaction with Mbd3 (Deshpande, Dai et al. 2009).

ChIP using Cdk2ap1 antibody successfully immunoprecipitated and amplified the *Oct4* promoter in Ebs derived from WT but not *Cdk2ap1*<sup>-/-</sup> mESCs. Because Cdk2ap1 lacks a DNA binding domain, it was hypothesized that the presence of Cdk2ap1 at the *Oct4* promoter

is mediated by its interaction with Mbd3. Following the introduction of an exogenous Cdk2ap1 in which the 8 amino acid sequence used to interact with Mbd3 was mutated, into *Cdk2ap1*<sup>-/-</sup> mESCs, ChIP data revealed that Cdk2ap1 was no longer present at the *Oct4* promoter, compared to when the WT form of Cdk2ap1 was used. This study presented valuable information about the role of Cdk2ap1 in epigenetic mechanisms during the differentiation of mESCs, and suggested that Cdk2ap1 may act as a “Velcro” that holds the different subunits of NuRD together at the promoter of *Oct4* during mESC differentiation and is indispensable for proper silencing of *Oct4*.

## **1.8. Conclusions and Proposed Directions:**

After considering the above mentioned studies which clearly demonstrated the importance of Cdk2ap1 in the self-renewal and differentiation of mESCs, our goal was to examine the role of CDK2AP1 in the self-renewal and differentiation of human ESCs. We proposed to investigate: the (a) effect of CDK2AP1 downregulation on the cell cycle and pluripotency of hESCs and (b) the effect of CDK2AP1 knockdown on the growth of human primary fibroblasts.

### **a. The effect of CDK2AP1 downregulation on the cell cycle and pluripotency of hESCs:**

Given that the deletion of Cdk2ap1 in mESCs resulted in persistent self-renewal along with a decrease in differentiation potential and inability to silence Oct4 upon the induction of differentiation (Deshpande, Dai et al. 2009), it would be interesting to test the effects of knockdown of CDK2AP1 on the cell cycle and pluripotency of hESCs.

Experiments under this specific aim attempted to investigate the role of CDK2AP1 in hESCs by creating stable cell lines in which CDK2AP1 is knocked down. After establishing the cell lines and confirming knockdown, a series of experiments were conducted to examine the

effects of this genetic manipulations on cell cycle, self-renewal and differentiation potential of the cells.

b. The effect of CDK2AP1 knockdown on primary human fibroblasts

Because the loss of CDK2AP1 has been associated with numerous types of cancers, we became interested in investigating the effect of CDK2AP1 knockdown on the growth of a normal primary human cell line. In addition, primary fibroblasts should serve as another potentially useful model to help us further understand the role of CDK2AP1 in human cells and if the role is different in embryonic stem cells when compared to differentiated cells.

In experiments proposed under this specific aim, a stable CDK2AP1 knockdown primary fibroblast cell line was generated, after which the effect of the knockdown on cell cycle and cell division was examined.

## **CHAPTER 2**

### **Knockdown of CDK2AP1 in Primary Human Fibroblasts Induces p53 Dependent Senescence**

#### **Abstract**

Cyclin Dependent Kinase-2 Associated Protein-1 (CDK2AP1) is known to be a tumor suppressor that plays a role in cell cycle regulation by sequestering monomeric CDK2, and targeting it for proteolysis. A reduction of CDK2AP1 expression is known to be a negative prognostic indicator in patients with oral squamous cell carcinoma and also associated with increased invasion in human gastric cancer tissue. CDK2AP1 overexpression was shown to inhibit growth, reduce invasion and increase apoptosis in prostate cancer cell lines. In this study, we investigated the effect of CDK2AP1 downregulation in primary human dermal fibroblasts. Using a short-hairpin RNA to reduce its expression in primary human fibroblasts, we found that knockdown of CDK2AP1 resulted in reduced proliferation and the induction of senescence associated beta-galactosidase activity. CDK2AP1 knockdown also resulted in a significant reduction in the percentage of cells in the S phase and an accumulation of cells in the G1 phase of the cell cycle.

BrdU proliferation assay revealed a significant reduction in proliferation of the CDK2AP1-knockdown fibroblasts. Immunocytochemical analysis also revealed that the CDK2AP1 knockdown fibroblasts significantly increased the percentage of cells that exhibited  $\gamma$ -H2AX foci, which could indicate presence of DNA damage. Quantitative PCR analysis also revealed that knockdown of CDK2AP1 resulted in an increase in the mRNA levels of *p53*, *p21*, and the p53-responsive apoptotic genes *BAX* and *PUMA*. In primary human fibroblasts in which p53 and CDK2AP1 were simultaneously downregulated, there was: (a) no increase in senescence associated beta-galactosidase activity, (b) decrease in the number of cells in the G1-phase and increase in number of cells in the S-phase of the cell cycle, and (c) decrease in the mRNA levels of *p21*, *BAX* and *PUMA* when compared with CDK2AP1 knockdown only fibroblasts. Altogether, our results show knockdown of CDK2AP1 in primary human fibroblasts reduced proliferation and induced premature senescence, with the observed phenotype being p53 dependent.

## Introduction

CDK2AP1 is a cell cycle regulator that controls the G1-S phase transition by negatively regulating CDK2 (Shintani, Ohshima et al. 2000). In vitro studies focused on overexpression of CDK2AP1 in prostate cancer cell lines results in a decrease in levels of CDK2 and its kinase activity, leading to an accumulation of cells in the G1 phase and a reduction in cells that are in the S phase of the cell cycle (Zolochovska, Figueiredo 2009). This outcome has been reasoned to be mediated by either the sequestration of monomeric CDK2 or by targeting it for proteolysis. Another mechanism by which CDK2AP1 regulates G1-S phase transition, is by directly binding

the DNA polymerase/alpha-primase complex and inhibiting the initiation step of DNA replication (Matsuo, Shintani et al. 2000). This inhibition may also be a result of CDK2AP1-mediated reduction in CDK2 activity, which is known to stimulate DNA replication by phosphorylating the DNA polymerase-alpha-primase complex.

CDK2AP1 has also been found to mediate the growth inhibitory effects of TGF- $\beta$  with studies in normal human keratinocytes treated with TGF- $\beta$ , increased cellular levels of CDK2AP1 mRNA and protein (Hu, Hu et al. 2004). Analysis of the results suggests that SMAD induced by TGF- $\beta$ 1 binds at the proximal promoter of the CDK2AP1 gene. A significant correlative expression of TGF- $\beta$  receptor II (TGF $\beta$ RII) and CDK2AP1 has been found in human oral squamous cell carcinoma (OSCC) tissues with an observed loss of expression of CDK2AP1 and p21 (Peng, Shintani et al. 2006). It has also been found that OSCC lines that were resistant to TGF- $\beta$ , were unable to induce SMADs and CDK2AP1, indicating a critical role for CDK2AP1 in mediating the growth inhibitory effects of TGF- $\beta$  (Peng, Shintani et al. 2006). The effects of overexpressing CDK2AP1 in prostate cancer cell lines, in which it is downregulated were also evaluated (Zolochevska, Figueiredo 2009). Overexpression of CDK2AP1 in prostate cancer cell lines lead to increased apoptosis, growth arrest and reduced invasion. In gastric cancer, it was found that patients who had higher levels of CDK2AP1 in their samples had a better prognosis than patients who had low levels of CDK2AP1 (Choi, Sohn et al. 2009).

In this study, we demonstrate a novel role for CDK2AP1 in primary human fibroblasts, with knockdown of CDK2AP1 resulting in reduced proliferation and p53-dependent senescence.

## **Materials and Methods**



## **Generation of Primary Human Fibroblasts Expressing CDK2AP1-specific shRNA and p53-specific shRNA**

Primary human dermal fibroblasts (HDF) (Coriell Cell Repositories, NJ) were routinely maintained in medium containing MEM, 15% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, with subculturing ratios of 1:4 using 0.05% Trypsin solution. All reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise noted.

We have identified two potent shRNAs targeted to CDK2AP1 mRNA. Multiple shRNAs were obtained from commercially available sources (Open Biosystems, PA; Sigma-Aldrich, MO) and screened for their effectiveness. Control scrambled sequences were used similarly. To identify the shRNA clone that produced the strongest knockdown of CDK2AP1, human fibroblasts were transduced with the different shRNA clones using lentiviral vectors, and successfully transduced cells were selected by puromycin treatment (3 µg/ml). Following 6 days of selection, antibiotic-resistant cells were harvested and RNA extracted. QPCR analyses of human CDK2AP1-specific primers were conducted. In our experiments, one shRNA (labeled as shRNA1 henceforth) (Open Biosystems, PA) produced the strongest knockdown and was used in subsequent experiments. A second validated CDK2AP1 shRNA (labeled as shRNA2 henceforth) (Sigma-Aldrich, MO) that target the 3'-UTR was also used in our studies. In experiments involving analysis of the role of p53, expression was downregulated using lentiviral delivery of p53-specific shRNA (Addgene, MA, USA), followed by validation of knockdown by qPCR analyses.

## **RNA Isolation, Real Time Reverse Transcription Polymerase Chain Reaction, and Gene Expression Analysis.**

**Table 1: Sequences of Forward and Reverse Primers Used in qPCR Analysis.**

Primer	Sequence
GAPDH Forward	TTGCCATCAATGACCCCTTCA
GAPDH Reverse	CGCCCCACTTGATTTTGA
CDK2AP1 Forward	ATGTCTTACAAACCGAACTGGC
CDK2AP1 Reverse	GCCCGTAGTCACTGAGCAG
CDKN1A (p21) Forward	TGTCCGTCAGAACCCATGC
CDKN1A (p21) Reverse	AAAGTCGAAGTTCCATCGCTC
TP53 Forward	CAGCACATGACGGAGGTTGT
TP53 Reverse	TCATCCAAATACTCCACACGC
PUMA Forward	GACCTCAACGCACAGTACGAG
PUMA Reverse	AGGAGTCCCATGATGAGATTGT
BAX Forward	CCCGAGAGGTCTTTTCCGAG
BAX Reverse	CCAGCCCATGATGGTTCTGAT

RNA was isolated using RNeasy kit (Qiagen, CA, USA), according to the manufacturer's protocols and quantified using BioMate3 UV-VIS Spectrophotometer (Thermo Scientific, MA, USA). Complimentary DNA (cDNA) was synthesized from 1 µg of RNA using cDNA reverse transcription kit (Applied Biosystems, CA). Gene expression within different samples was analyzed using quantitative real time RT-PCR (QPCR). QPCR was performed in an ABI HT7900 system (Applied Biosystems, CA) and the data were acquired using sequence detection system software (SDS v2.2.1, Applied Biosystems, CA). Gene expression data (three replicates) were acquired and SDS software was used to estimate differential gene expression using  $\Delta$ CT quantification methods. Endogenous *Gapdh* was used for normalization. Commercially available primers for *Cdk2ap1* were obtained from Origene, MD, while other primers summarized in Table 1 were obtained from Integrated DNA Technologies, IA.

### **Antibodies and Immunocytochemical Analysis**

Under different experimental conditions, human fibroblasts were seeded onto four chambered glass slides. Paraformaldehyde (PFA, 4%) in PBS was used for fixation, permeabilization for intracellular markers was achieved with 0.2% Triton X-100 in PBS and blocked with normal goat serum at room temperature. Fixed cells were incubated with primary antibodies: CDK2AP1 (Santa Cruz, CA), Anti-phospho-Histone H2AX (Millipore, CA). Goat anti-mouse IgG conjugated to Alexa 488 (for  $\gamma$ -H2AX staining) and Goat anti-rabbit IgG conjugated to Alexa 594 (for CDK2AP1 staining) (Invitrogen, CA,) were used as secondary antibodies. The samples were prepared for imaging by overnight exposure to secondary antibody at 4°C followed by PBS washes, nuclear stain using DAPI (4',6-diamidino-2-phenylindole) and finally mounting the slides. Fluorescent images were acquired using a CoolSnap EZ camera

(Photometrics, Tucson, AZ) mounted on a Nikon Eclipse TE 2000-S inverted microscope (Nikon, Melville, NY) with attached image analysis software. All image settings were controlled for uniform acquisition between samples. Specifically, a uniform exposure time was maintained for images acquired from experimental samples as well as negative controls for background subtraction. Percentages of  $\gamma$ -H2AX positive cells were calculated in multiple fields per sample (n=28) by counting the number of cells containing at least 3 foci in their nucleus and dividing by the total number of cells in each field of interest.

### **Bromodeoxyuridine (BrdU) Proliferation Assay**

For proliferation assays, fibroblasts under different experimental conditions in four-chambered glass slides were propagated overnight, with subsequent incubation in medium containing 20 $\mu$ g/ml Bromodeoxyuridine (BrdU) for a period of 2 hrs. Cells were then washed with ice cold PBS buffer and fixed with 100% methanol at -20°C for 15 min. After fixation, cells were washed in cold PBS and permeabilization achieved with 0.2% Triton X-100 in PBS. For DNA denaturation (antigen retrieval), cells were treated with 2N HCl solution for 45 min at room temperature. To neutralize the HCl solution, Borate buffer was added for 6 min and cells washed. Blocking was achieved by treatment with 3% goat serum in medium for 45 min at room temperature. Fixed cells were incubated with Anti-BrdU (Abcam, MA) as the primary antibody. Goat anti-rat IgG conjugated to Alexa 594 (Invitrogen, CA) was used as a secondary antibody. The samples were prepared for imaging by overnight exposure to secondary antibody at 4°C followed by PBS washes, nuclear stain using DAPI (4',6-diamidino-2-phenylindole) and finally mounting the slides. To determine the percentage of BrdU positive cells, slides were viewed at 20X magnification and random fields chosen in which total number of cells was determined by

counting DAPI, followed by counting the BrdU-positive cells. Triplicate counts of at least 500 cells each were acquired and the percentage of BrdU positive cells was determined by dividing the number of BrdU positive cells by total number of cells.

### **Cell Cycle Profile Analysis**

Cells to be analyzed were trypsinized, washed, stained with propidium iodide for 45 min at 37°C, filtered through a 30 µm mesh to eliminate clumps and subjected to cell cycle analysis on a Accuri™ C6 Flow Cytometer (BD Biosciences, CA). Data were analyzed using the software provided by the manufacturer and samples analyzed in triplicate.

### **Senescence Associated $\beta$ -galactosidase Assay**

Cells to be assayed were seeded in 6-well plates for the senescence-associated  $\beta$ -galactosidase assay. After 24 h, cells were fixed and stained with X-gal for detection of  $\beta$ -galactosidase activity using a senescence  $\beta$ -galactosidase staining kit (Cell Signaling, MA). After 24 h, cells exhibiting positive  $\beta$ -galactosidase activity (turned blue) at pH 6.0 were counted under a light microscope. The percentage of cells exhibiting positive staining was determined in 10 individual fields of interest.

### **Western Blotting**

Cells for analysis were harvested by trypsinization, centrifuged at 1000 rpm and washed once with ice-cold PBS buffer. Cells were then lysed using the Total Protein Extraction Kit (EMD Millipore, MA) based on protocol provided by the manufacturer. Cell lysates were then subjected to Western blot analyses using specific antibodies to various cyclins. Cell lysates were prepared from wild type and CDK2AP1 knockdown primary human fibroblasts and analyzed for

p53, p21 and  $\beta$ -tubulin expression by Western blot using specific antibodies. p53 and p21 antibodies were obtained from Cell Signaling, MA, while  $\beta$ -tubulin antibody was obtained from Developmental Studies Hybridoma Bank, IA. Appropriate infrared emitting-conjugated secondary antibodies were obtained from Invitrogen, CA. Detection was then carried out using the Odyssey Infrared Imaging System (Li-Cor Biosciences, NE)..

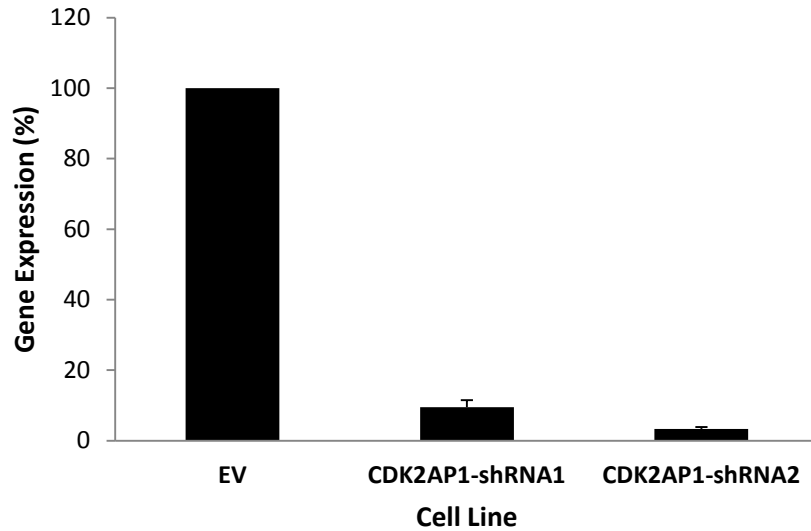
## **Results**

### **Knockdown of CDK2AP1 in Primary Human Fibroblasts Results in Decreased Cell Proliferation and Alters Cell Cycle Profile**

To investigate the effect of CDK2AP1 knockdown on the growth of primary human fibroblasts, we used lentiviral delivery of shRNA1 to downregulate CDK2AP1 in primary human dermal fibroblasts. CDK2AP1 expression was then examined using qPCR (Figure 3) and immunocytochemistry (Figure 4). Based on qPCR analysis, we were able to achieve 90% knockdown of CDK2AP1, with statistical analysis indicating significant differences ( $p < 0.05$ ) in the expression patterns between primary human fibroblasts transduced with the shRNA1 and normal fibroblasts (Figure 3). Additional transduction with a second different CDK2AP1 shRNA (shRNA2) resulted in a 96% knockdown of CDK2AP1 (Figure 3). Following the generation of CDK2AP1 knockdown primary human fibroblasts, we examined the effect of the knockdown on cell proliferation. Initial analyses conducted in triplicate using hemocytometer counting cells indicated that CDK2AP1 knockdown cells exhibited an extremely slow rate of proliferation, suggesting that depletion of CDK2AP1 inhibited cell proliferation (Figure 5). Subsequent analysis using the BrdU proliferation assay indicated that the CDK2AP1 knockdown cells had a

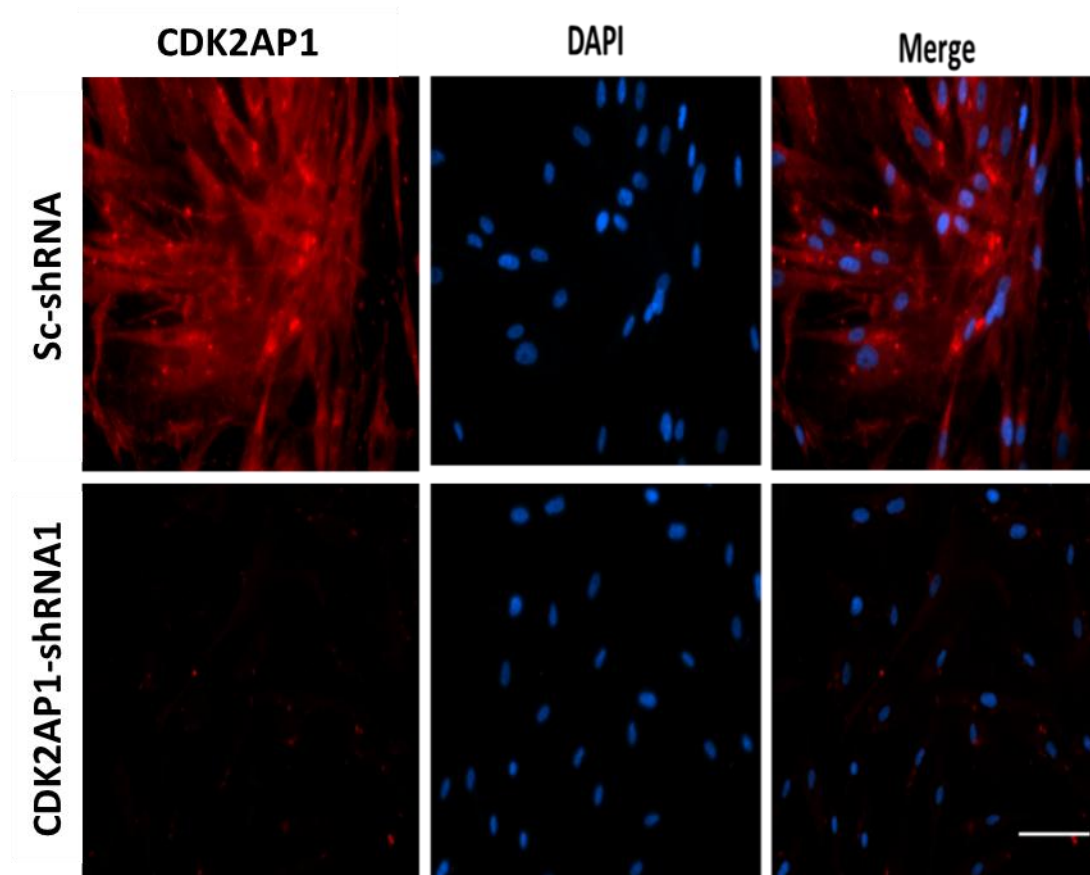
significantly lower BrdU incorporation than the wild type fibroblasts (p-value = 0.002) (Figure 6). Taken together, these results suggest that the knockdown of CDK2AP1 in primary human fibroblasts decreases proliferation.

Cell cycle analysis was then conducted on an equal number of HDFs transduced with an empty vector (EV) or HDFs transduced with CDK2AP1 shRNA1 or HDFs transduced with CDK2AP1 shRNA2. Analysis demonstrated that the knockdown of CDK2AP1 significantly increased cells in the G1 phase of the cell cycle from 78% in the control cells to 94% in CDK2AP1 shRNA1 transduced cells (p-value = 0.00026) to 95% in CDK2AP1 shRNA2 transduced cells (p-value = 0.00022). In addition, knockdown resulted in a significant decrease in the percentage of cells in the S-phase of the cell cycle from 8% in the control cells to 1.3% in the CDK2AP1-shRNA1 transduced cells (p-value = 0.005) and to 0.6% in the cells transduced CDK2AP1-shRNA2 (p-value = 0.004). Under each condition, we also observed few necrotic/apoptotic cells present in the sub G0/G1 phase of the cell cycle with 7% in the control sample, and 3% and 4% in the CDK2AP1-shRNA1 and CDK2AP1-shRNA2 transduced cells, respectively. Taken together, these results indicate that the depletion of CDK2AP1 prevents progression from the G1 to the S phase of the cell cycle.

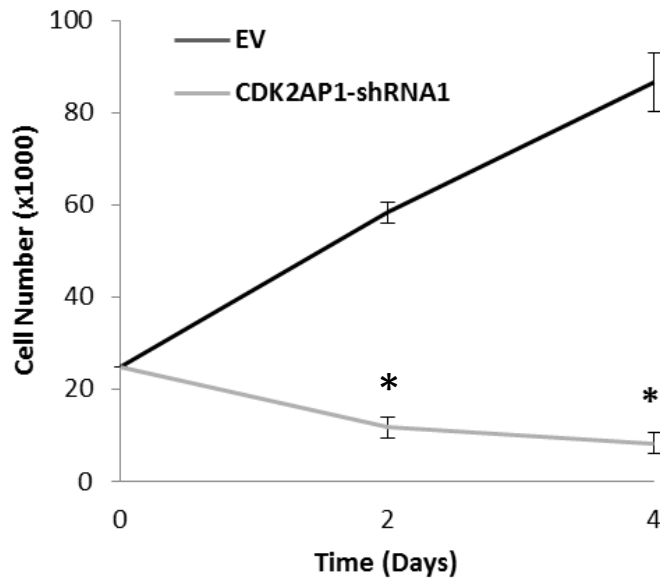


**Figure 3: *CDK2AP1* Gene Expression was Significantly Reduced in HDFs that were Transduced with Specific shRNAs.** Primary HDFs were transduced with either an empty vector (EV) or with a CDK2AP1-shRNA1 or CDK2AP1-shRNA2. After 6 days of puromycin selection at 3 $\mu$ g/ml, total RNA was extracted and real-time qPCR was used to measure the expression of *CDK2AP1*. The cells that were transduced with CDK2AP1-shRNA1 had 90% reduction in *CDK2AP1* expression and cells that were transduced with CDK2AP1-shRNA2 had 96% reduction, when compared to EV transduced cells. Results are presented together with standard deviation from experiments conducted in triplicate.



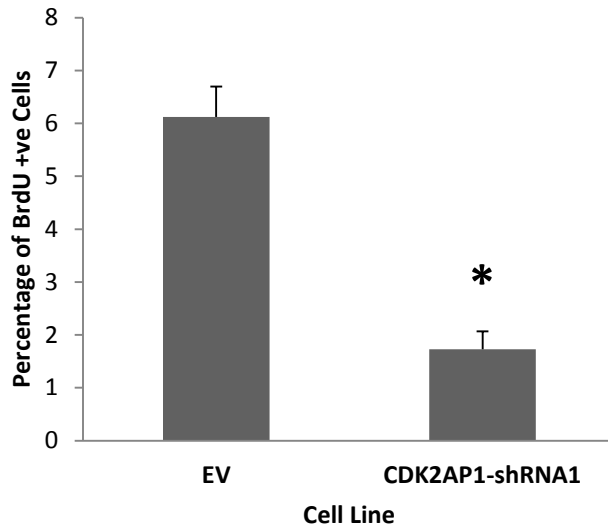


**Figure 4: CDK2AP1 Protein is Successfully Downregulated in Cells Transduced with the CDK2AP1 Specific shRNA1.** Primary HDFs were transduced with either a scrambled shRNA (sc-shRNA) (top panel) or with a CDK2AP1-shRNA1 (bottom panel). Following antibiotic selection, equal number of cells was seeded onto chamber slides, allowed to propagate for 24 h, fixed and stained with CDK2AP1 specific antibody (red) and DAPI (blue-nuclear stain). Immunocytochemical staining data showed successful knockdown of CDK2AP1 in primary HDFs. Scale bar represents 100  $\mu$ m.

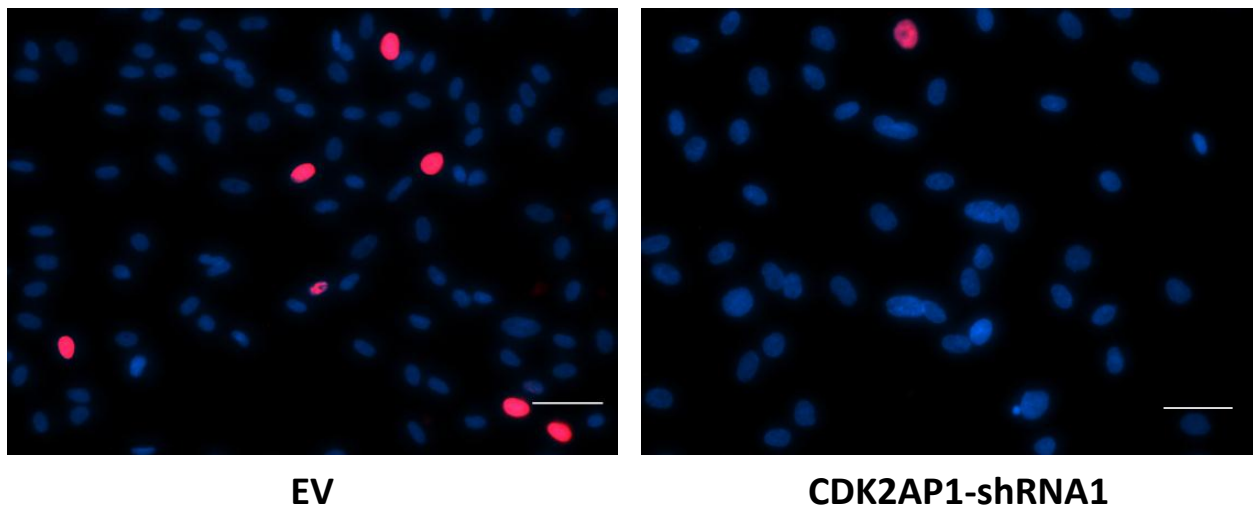


**Figure 5: Knockdown of CDK2AP1 in Primary HDFs Reduces Proliferation.** Twenty-five thousand cells that were either transduced with an empty vector (EV) or CDK2AP1 shRNA1 were seeded per well in a 24-well plate. Cells were harvested and counted in triplicates at 2 and 4 days post seeding (\*- p-value < 0.05. Comparisons were made between EV and CDK2AP1 shRNA1 transduced cells at respective time points). Results are presented together with standard deviation from experiments conducted in triplicate.

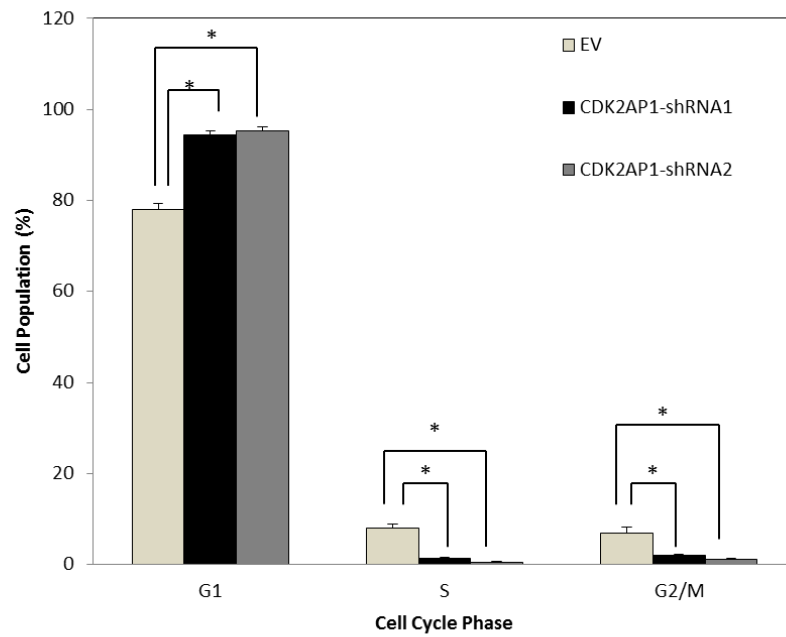
A.



B.



**Figure 6: Knockdown of CDK2AP1 in Primary HDFs Reduces BrdU Incorporation.** A. Cells transduced with an empty vector (EV) or CDK2AP1-shRNA1 were labeled with 20  $\mu$ M BrdU for 2 h, fixed and stained with a BrdU specific antibody and the percentage of BrdU positive cells was calculated. Results are presented together with standard deviation from experiments conducted in triplicate. (\*-p-value < 0.05). B. Showing representative pictures of the quantified immunocytochemistry. BrdU positive cells are shown in red, nuclei were stained with DAPI (blue). Scale bar represent 50  $\mu$ m.



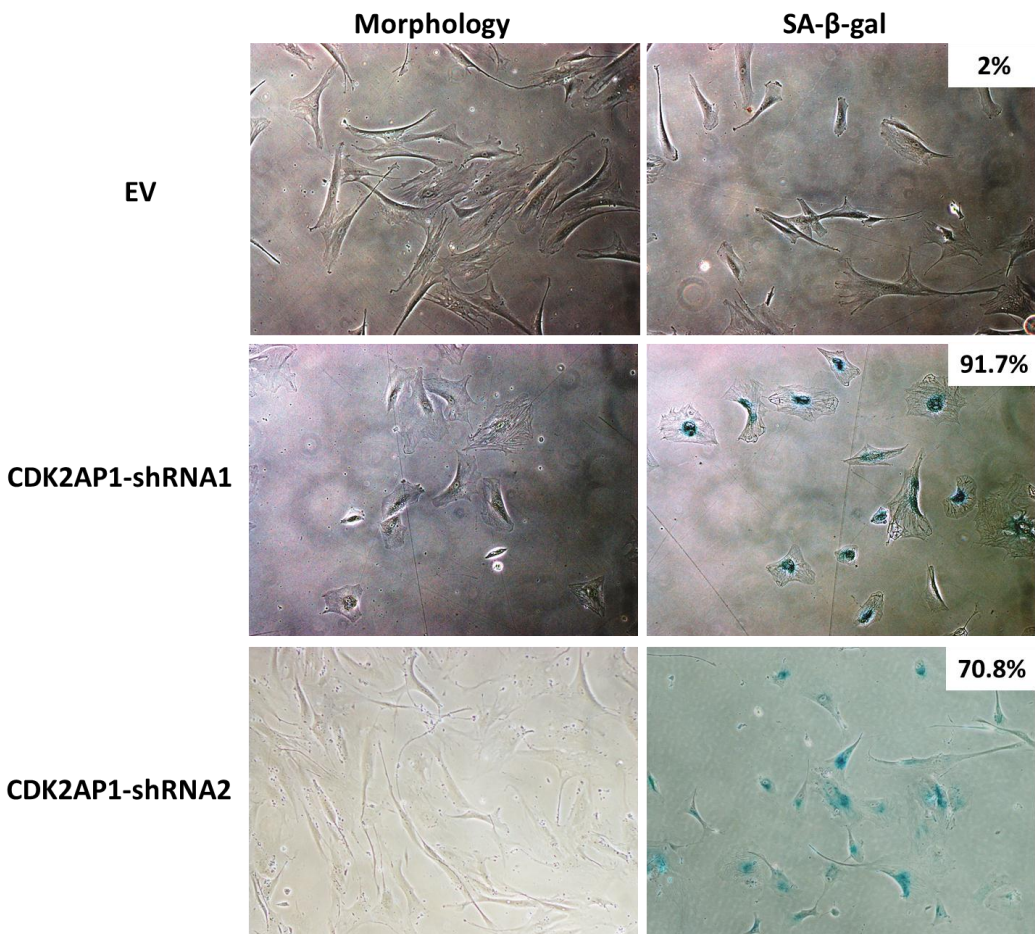
**Figure 7: Knockdown of CDK2AP1 Reduces the Percentage of Cells in the S and G2/M Phases and Increases Cells in the G1 Phase of the Cell Cycle.** Cells that were transduced with empty vector (EV) or with CDK2AP1-shRNA1 or with CDK2AP1-shRNA2 were harvested and equal numbers stained with propidium iodide (PI) and analyzed using an Accuri C6 flow cytometer. Results are presented together with standard deviation from experiments conducted in triplicate (\*-p-value < 0.05).

### **Knockdown of CDK2AP1 in Primary Human Fibroblasts Induces Premature Senescence**

During the period of monitoring cell viability, we observed that CDK2AP1 knockdown fibroblasts displayed a flattened cellular morphology with progressive cell passage number. To investigate whether CDK2AP1 knockdown leads to growth arrest, HDFs transduced with an empty vector or HDFs transduced with CDK2AP1-shRNA1 or HDFs transduced with CDK2AP1-shRNA2, were plated for senescence associated  $\beta$ -galactosidase assay (SA- $\beta$ -gal). Detection of  $\beta$ -galactosidase activity at pH 6.0 is a known characteristic of senescent cells (Dimri, Lee et al. 1995). After 24 h, cells were fixed and stained with X-gal for the detection of  $\beta$ -galactosidase activity (Figure 8). 91.7% of HDFs transduced with CDK2AP1-shRNA1 and 70.8% of CDK2AP1-shRNA2 transduced cells displayed appearance of enlarged blue cells in contrast to the control EV transduced cells, which failed to exhibit a detectable blue appearance. Therefore, these results suggest that knockdown of CDK2AP1 leads primary human fibroblasts to undergo premature senescence.

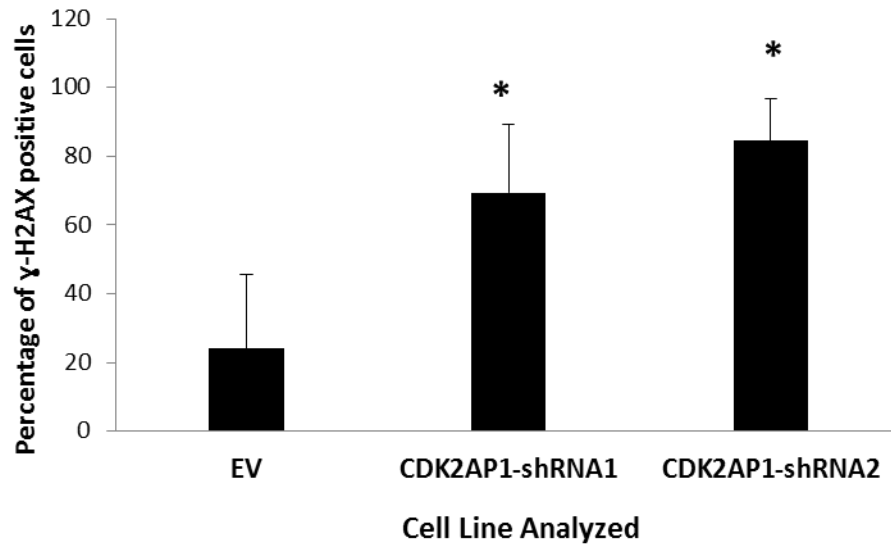
### **Knockdown of CDK2AP1 in Primary Fibroblasts Increases DNA Damage**

To ascertain the reason behind the unexpected reduced proliferation of the CDK2AP1 knockdown HDFs, we hypothesized that CDK2AP1 knockdown might contribute to a temporary abnormal increase in the progression into S-phase of the cell cycle and hence, an uncontrolled increase in DNA synthesis. This is based on the reported function of CDK2AP1 as a negative regulator of CDK2 and we expected that this could be contributing to errors in DNA synthesis and DNA damage (Shintani, Ohyama et al. 2000).

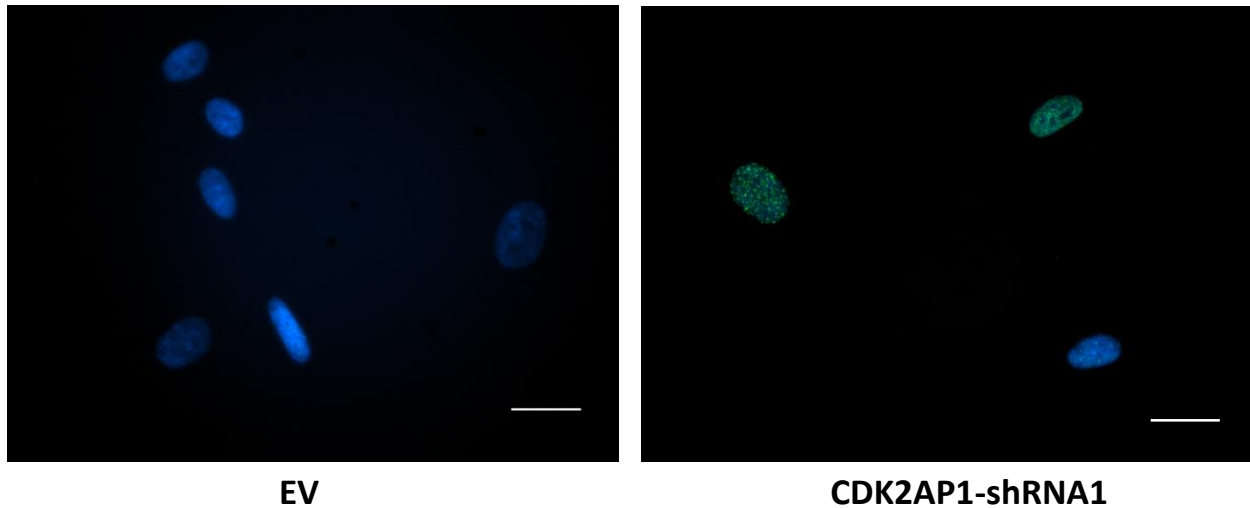


**Figure 8: Knockdown of CDK2AP1 Altered Morphology and Exhibited Senescence-Associated  $\beta$ -galactosidase Expression.** HDFs that were transduced with an empty vector (EV) or CDK2AP1-shRNA1 or CDK2AP1-shRNA2, were seeded and grown in a 6-well plate and senescence-associated  $\beta$ -galactosidase assay was performed. CDK2AP1 knockdown fibroblasts displayed a significantly higher senescence associated  $\beta$ -galactosidase activity. The percentage of cells exhibiting  $\beta$ -galactosidase activity is shown at the top of each panel in the right.

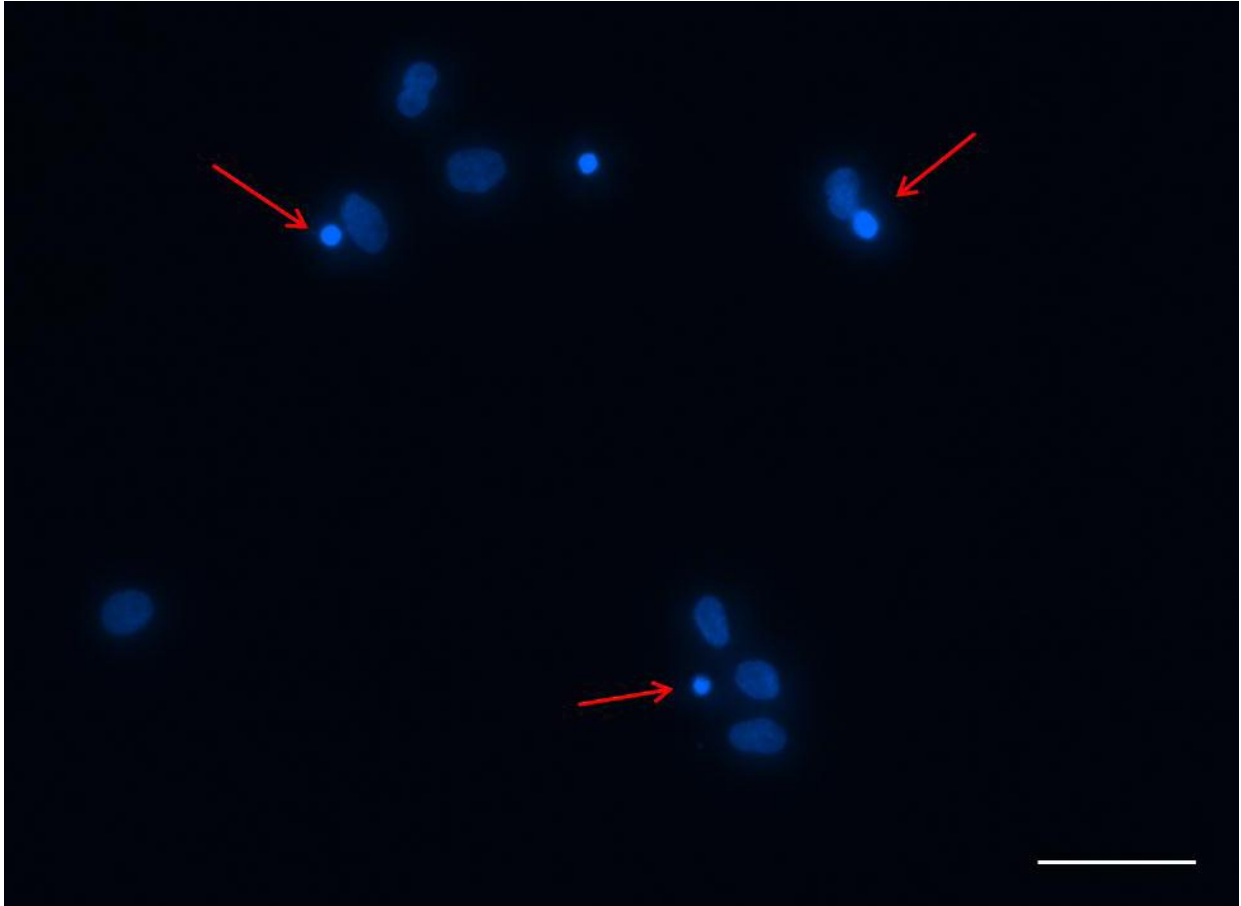
**A.**



**B.**



**Figure 9: Knockdown of CDK2AP1 in Primary HDFs Increases  $\gamma$ -H2AX Signal.** A. Primary HDFs were either transduced with empty vector (EV), CDK2AP1-shRNA1 or CDK2AP1-shRNA2. Following antibiotic selection, immunocytochemistry was carried out using a  $\gamma$ -H2AX specific antibody. Percentage of  $\gamma$ -H2AX was calculated in multiple (n=28) randomly selected fields by dividing the number of  $\gamma$ -H2AX positive cells by total number of cells in the field. Results are presented together with standard deviation from multiple fields of view (\*-p-value < 0.05). B. Example of EV and CDK2AP1-shRNA1 cells stained with  $\gamma$ -H2AX (green) and the nuclear dye, DAPI (blue). Scale bar represents 50  $\mu$ m.



**Figure 10: Knockdown of CDK2AP1 in Primary HDFs Leads to Increased Micronuclei Formation.** Red arrows point to micronuclei, which may be a sign of genetic instability in these cells. Scale bar represents 100  $\mu\text{m}$ .



To examine this possibility, we measured the number of  $\gamma$ -H2AX foci in equal numbers of CDK2AP1-shRNA1, CDK2AP1-shRNA2 or EV transduced cells that were seeded onto 4-chamber slides and stained using  $\gamma$ -H2AX antibody. We found that the knockdown of CDK2AP1 in HDFs resulted in a significant increase in  $\gamma$ -H2AX foci with 70% of the CDK2AP1-shRNA1 HDFs and 84.5% CDK2AP1-shRNA2 cells staining positive, while only 25% of EV cells were positive, indicating a potential increase in DNA damage upon CDK2AP1 knockdown (Figure 9). Additionally, nuclear staining with DAPI dye indicated that the CDK2AP1-knockdown cells exhibited a high number of micronuclei that were surrounding the nuclei of the cells; a phenomenon not observed in the control fibroblasts (Figure 10). This observation might be considered evidence of genomic instability that the knockdown fibroblasts might have experienced before they entered senescence.

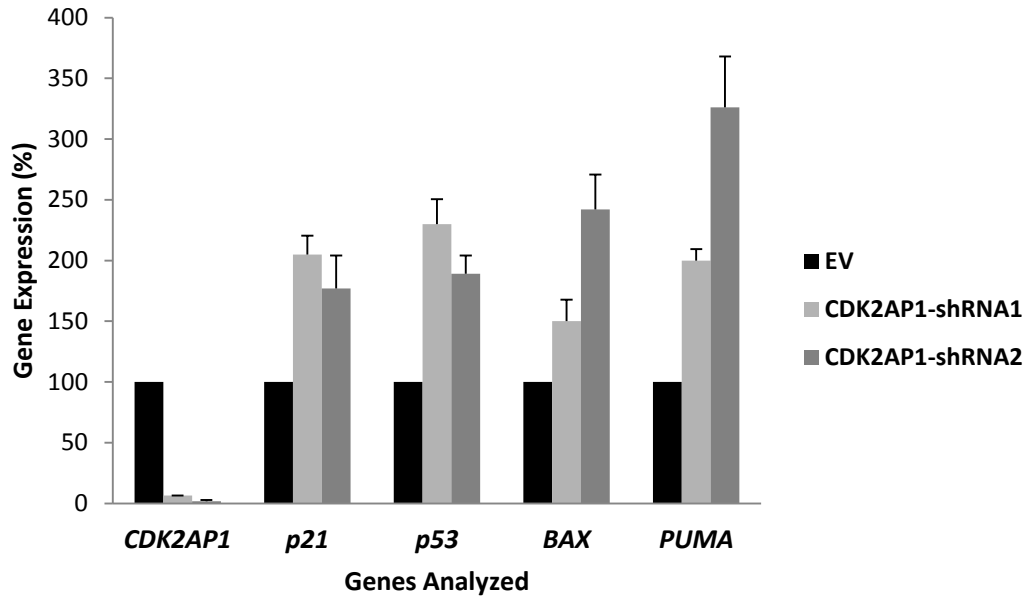
### **Knockdown of CDK2AP1 Increases the Expression of p53, p21 and the Apoptotic Genes (*BAX* and *PUMA*).**

CDK2AP1 is known to be a member of the Nucleosome Remodeling and Deacetylation (NuRD) Complex (Spruijt, Bartels et al. 2010), which is a repressive complex responsible for epigenetic regulation of numerous genes. Downregulation of members of the NuRD complex has been previously associated with the transcriptional activation of p53 (Kai, Samuel et al. 2010). Therefore, we speculated that the knockdown of CDK2AP1 may lead to aberrant epigenetic regulation of the p53 gene, leading to abnormal increase in its transcription. QPCR analysis (Figure 11) indicated that *p53* mRNA levels was increased by 2.3 fold in the CDK2AP1-shRNA1 HDFs and by 1.89 fold in the HDFs transduced with CDK2AP1-shRNA2 when

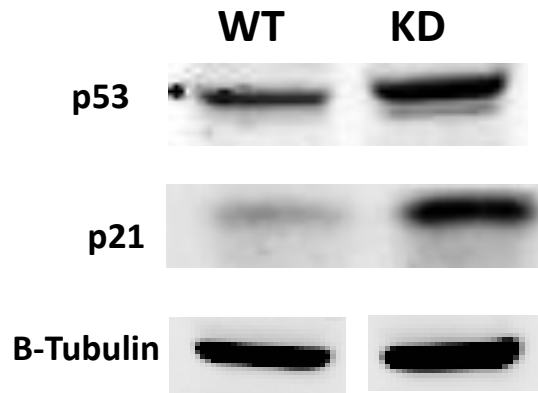
compared with EV transduced cells. Similarly, we observed that *p21* mRNA levels were increased by 2.1 fold in the CDK2AP1-shRNA1 cells and by 1.8 fold in the CDK2AP1-shRNA2 when compared with EV transduced cells. Due to observed decrease in cell viability upon CDK2AP1 knockdown, we also decided to examine the expression of candidate p53-responsive apoptotic genes, *BAX* and *PUMA*. QPCR analysis indicated that *BAX* mRNA levels was increased by 1.5 fold in the CDK2AP1-shRNA1 transduced HDFs and by 2.4 fold in the CDK2AP1-shRNA2 when compared with EV transduced cells. Similarly, we observed that *PUMA* mRNA levels were increased by 2.0 fold in the CDK2AP1-shRNA1 cells and by 3.6 fold in cells transduced with CDK2AP1-shRNA2 when compared with EV cells. Western blot analysis further confirmed the increase in p53 and p21 protein levels (Figure 12).

### **Observed Senescence in CDK2AP1 Knockdown Primary HDFs is p53 Dependent**

Following CDK2AP1 knockdown, we have observed a ~ 2 fold increase in *p53* mRNA levels (Figure 11) which was further confirmed by observed increase in p53 protein levels by Western blot analysis (Figure 12). The increase in p53 levels may have resulted in the observed increase of the transcription of *p21*, *BAX* and *PUMA* (Figure 11). To investigate if the observed increase in the levels of transcription of the aforementioned genes in the CDK2AP1 knockdown primary HDFs is p53 dependent, we co-transduced primary HDFs with p53 and CDK2AP1 shRNAs and assayed *CDK2AP1*, *p53*, *p21*, *BAX* and *PUMA* expression by qPCR analysis. The double-knockdown cells exhibited significantly lowered expression of *p53*, *p21*, *BAX* and *PUMA* (p-value <0.05) when compared with CDK2AP1 only knockdown cells, thus suggesting that the



**Figure 11: Knockdown of CDK2AP1 in Primary HDFs Increases mRNA Levels of *p53*, *p21*, *BAX* and *PUMA*.** To investigate if CDK2AP1 knockdown affects the expression of *p53*, *p21* and the apoptotic genes *BAX* and *PUMA*, total RNA was extracted from cells transduced with empty vector (EV), cells transduced with CDK2AP1-shRNA1 and cells transduced with CDK2AP1-shRNA2, and levels of *p53*, *p21*, *BAX* and *PUMA* were measured by qPCR. Results are presented together with standard deviation from experiments conducted in triplicate. Knockdown of CDK2AP1 using two different shRNAs increased the transcription of *p53*, *p21*, *BAX* and *PUMA*. (p-value < 0.05. Comparisons were made between EV and CDK2AP1-shRNA1 or EV and CDK2AP1-shRNA2 transduced cells).

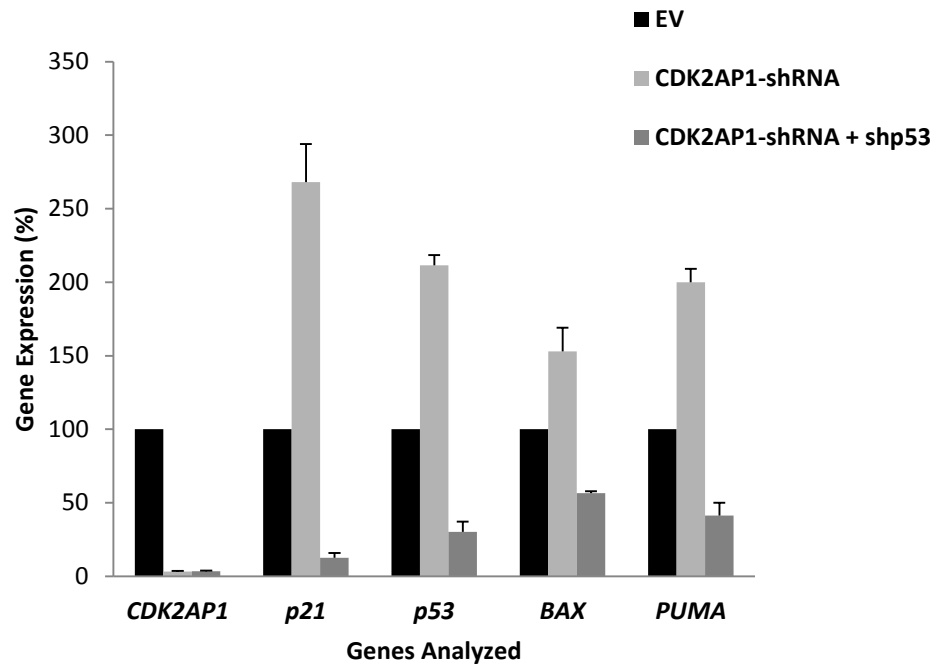


**Figure 12: Knockdown of CDK2AP1 in HDFs Increases p53 and p21 Protein Levels.** Whole cell lysates were extracted from HDFs transduced with an empty vector (WT) or with CDK2AP1 shRNA 1 (KD) and analyzed by Western Blot. Knockdown of CDK2AP1 in primary HDFs increased protein levels of p53 and p21.

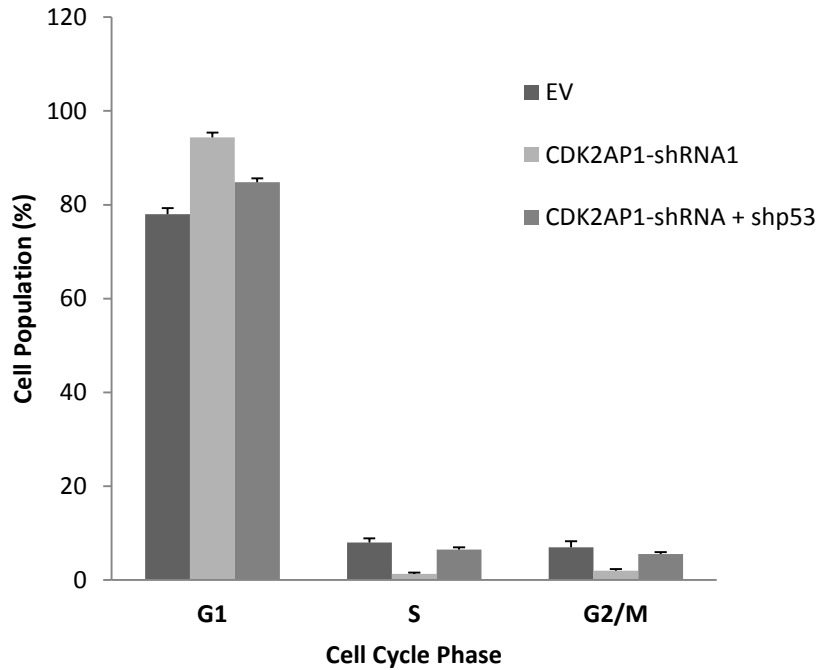
observed increase in *p21*, *BAX* and *PUMA* transcription levels in the CDK2AP1 only knockdown cells is p53 dependent.

In experiments conducted with primary HDFs, we found that knockdown of CDK2AP1 resulted in increased percentage of cells in the G1 phase of the cell cycle and prevents progression from the G1 to the S phase of the cell cycle (Figure 7). To test dependence on p53 expression, we examined the cell cycle profile of primary HDFs that were co-transduced with p53 and CDK2AP1 shRNAs. We found that the knockdown of p53 significantly increased the percentage of cells in the S phase (p-value = 0.0007) and reduced the cells in G1 phase of the cell cycle (p-value = 0.0004) (Figure 14) when compared to CDK2AP1 knockdown only primary HDFs (Figure 7).

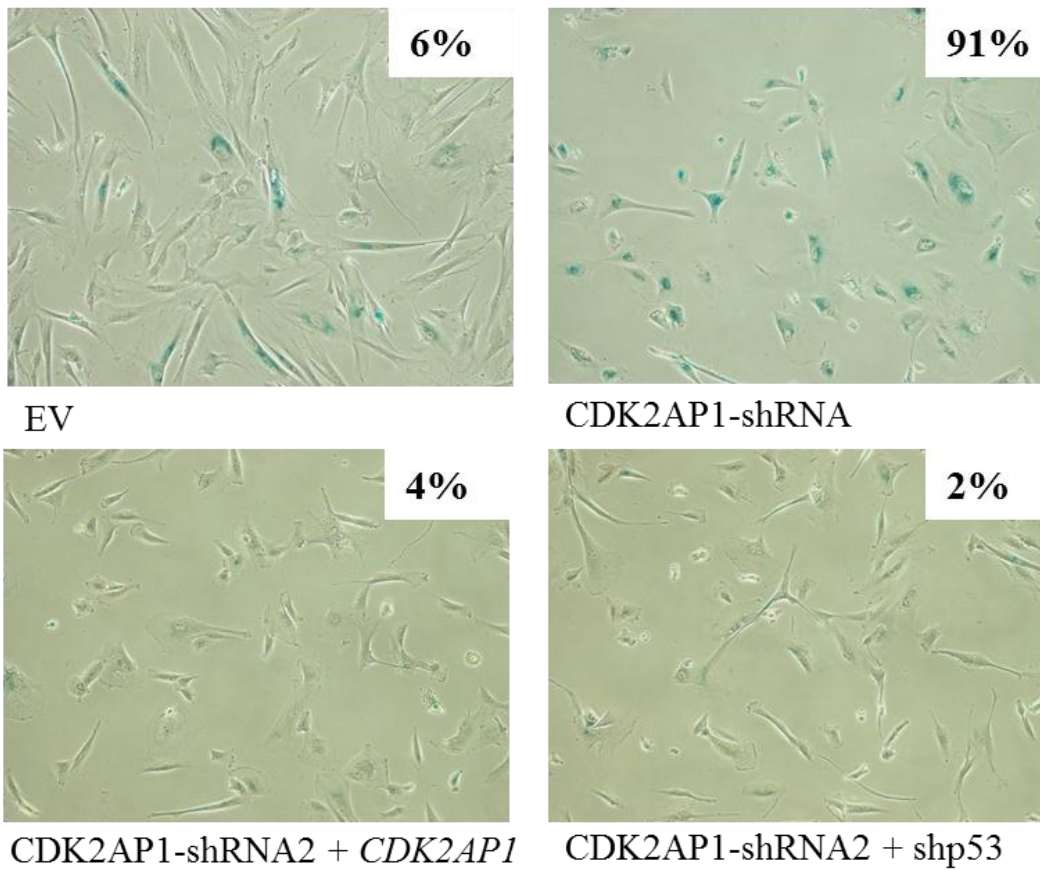
To investigate if the simultaneous knockdown of p53 and CDK2AP1 in primary HDFs would prevent premature senescence, HDFs transduced with an empty vector or HDFs transduced with CDK2AP1 shRNA1 or HDFs transduced with CDK2AP1 shRNA2 and exogenous *CDK2AP1* (CDK2AP1-shRNA2 + *CDK2AP1*) or HDFs transduced with CDK2AP1 shRNA2 and p53 shRNA (CDK2AP1-shRNA2 + shp53) were plated for senescence associated  $\beta$ -galactosidase assay (SA- $\beta$ -gal). After 48 h, cells were fixed and stained with X-gal for the detection of  $\beta$ -galactosidase activity (Figure 15). Our results indicate that the fibroblasts that were co-transduced with the CDK2AP1 and p53 shRNAs did not enter premature senescence and had extremely low  $\beta$ -galactosidase activity. Taken together, these results indicate that downregulation of CDK2AP1 increases p53 expression which pushes primary human fibroblasts to enter a p53 dependent premature senescence.



**Figure 13: Knockdown of p53 in Primary HDFs Prevents the CDK2AP1-knockdown Induced Increase in *p21*, *BAX* or *PUMA* gene expression.** Primary HDFs were transduced with empty vector (EV), CDK2AP1 shRNA or co-transduced with CDK2AP1 and p53 shRNAs. The gene expression of *CDK2AP1*, *p21*, *p53*, *BAX* and *PUMA* was analyzed in those cells by qPCR. We found that down regulating p53 with CDK2AP1 prevented the CDK2AP1-knockdown induced increase in *p21*, *p53*, *BAX* and *PUMA* levels ( $p < 0.05$ . Comparisons were made between EV and CDK2AP1-shRNA or CDK2AP1-shRNA and CDK2AP1-shRNA + shp53 transduced cells for each gene analyzed). Results are presented together with standard deviation from experiments conducted in triplicate.



**Figure 14: Simultaneous Knockdown of CDK2AP1 and p53 Results in Higher Percentage of Cells in the S Phase.** Primary HDFs were co-transduced with CDK2AP1 and p53 shRNAs, stained with propidium iodide and analyzed by flow cytometry to examine their cell cycle profile. Knockdown of CDK2AP1 in primary HDFs reduces the percentage of cells in the S and G2/M phases and increases cells in the G1phase of the cell cycle. When p53 is simultaneously downregulated with CDK2AP1, it results in an increase in the percentage of cells in the S-phase and reduction in the percentage of cells in the G1-phase of the cell cycle. Results are presented together with standard deviation from experiments conducted in triplicate. Cell cycle profiles of EV and CDK2AP1-shRNA1 transduced cells from figure 7 are also presented here to facilitate comparison.



**Figure 15: Simultaneous Knockdown of CDK2AP1 and p53 Prevents Premature Senescence.** Primary HDFs that were transduced with an empty vector (EV), CDK2AP1 shRNA1, CDK2AP1 shRNA2 and exogenous *CDK2AP1* (CDK2AP1-shRNA2+ *CDK2AP1*) or CDK2AP1 shRNA2 and p53 shRNA (CDK2AP1-shRNA2+shp53) were seeded onto a 6-well plate and senescence-associated β-galactosidase assay was performed. CDK2AP1 knockdown fibroblasts displayed a significantly higher senescence associated β-galactosidase activity, while the simultaneous knockdown of CDK2AP1 and p53 results in significantly reduced senescence associated β-galactosidase activity. The percentage of cells exhibiting β-galactosidase activity is shown at the top of each panel.



## Discussion and Conclusions

The function of CDK2AP1 as initially assessed in multiple cancer cell lines indicates its role in regulation of apoptosis, proliferation and invasion (Zolochavska, Figueiredo 2009). Although the primary function of CDK2AP1 as a cell cycle regulator that controls the G1-S phase transition by negatively regulating CDK2 has been ascertained (Shintani, Ohyama et al. 2000), its role in primary human fibroblasts has not been studied. To investigate the function of CDK2AP1 in primary human fibroblasts, we knocked down CDK2AP1 expression in primary human dermal fibroblasts (HDFs) using short hairpin RNA. Using a lentiviral approach, we were able to downregulate CDK2AP1 by ~90%. Following knockdown of CDK2AP1 in primary HDFs, we observed a distinct reduction in the proliferation potential of these cells, with the cells exhibiting morphological changes as well. The CDK2AP1 knockdown HDFs appeared enlarged, flat and more spread out. Given that reduced proliferation and flattened morphologies is a sign of premature senescence in primary somatic cells, we decided to examine the CDK2AP1 knockdown HDFs for senescence associated  $\beta$ -galactosidase activity. Results indicated that CDK2AP1 knockdown HDFs displayed a significantly higher senescence associated  $\beta$ -galactosidase activity.

Given that CDK2AP1 is a known inhibitor of CDK2 and has also been reported to associate with the DNA polymerase/alpha primase complex (Shintani, Ohyama et al. 2000, Matsuo, Shintani et al. 2000), we expected that following its knockdown, the inhibition might have been alleviated allowing abnormal increase in DNA synthesis, which could lead to DNA damage. Using immunocytochemical analysis, we examined the CDK2AP1 knockdown cells for levels of  $\gamma$ -H2AX foci, a known indicator of DNA damage. Our results indicated that the

CDK2AP1 knockdown HDFs had significantly higher number of cells that were  $\gamma$ -H2AX positive (having more than three  $\gamma$ -H2AX foci in each cell). Additionally, we observed that the CDK2AP1 knockdown HDFs had a high number of micronuclei that were surrounding the nuclei when examined by DAPI staining (Figure 10). These findings might be considered evidence of genomic instability that the CDK2AP1 knockdown HDFs might have experienced before they entered senescence. In addition, the suspected initial uncontrolled increase in DNA synthesis due to CDK2AP1 knockdown, might have been sensed as oncogenic stress by the cell, leading to the activation of the tumor suppressor p53 (Sakaguchi, Herrera et al. 1998)(Haigis, K.M., et al. 2011). Indeed, we have found p53 protein levels to be increased following CDK2AP1 knockdown. Also, following CDK2AP1 knockdown, CDK2 activity might have been increased causing increased phosphorylation of the retinoblastoma protein (pRb) leading to aberrant upregulation of the oncogene, E2F. This may have led to a p16 mediated degradation of the negative regulator of p53, Mouse Double Minute-2 (MDM2) (Sherr 2001) and p53 stabilization, which in turn could have resulted in senescence. Additionally, our results also demonstrate an increase in *p21* and the candidate p53-responsive apoptotic genes *BAX* and *PUMA* expression in the CDK2AP1 knockdown HDFs.

CDK2AP1 has also been reported to be a member of the epigenetically repressive complex, NuRD (Spruijt, Bartels et al. 2010). A previous study demonstrated that disruption of a member of the NuRD complex leads to an increase in p53 levels (Kai, Samuel et al. 2010). In our studies, we have observed an increase in *p53* transcription following CDK2AP1 knockdown, which could be a result of the disruption of the NuRD complex. Deletion of CDK2AP1 has been previously reported to alter epigenetic marks on genes that are regulated by the NuRD complex,

and these genes could not be silenced in the absence of CDK2AP1 due to disrupted localization of NuRD (Deshpande, Dai et al. 2009). Therefore, it is possible that following CDK2AP1 knockdown, the NuRD complex could not localize properly to regulate *p53* transcription, which could thus have caused the observed increased levels of *p53* in the CDK2AP1 knockdown HDFs.

Given the observed increase in p53 levels upon CDK2AP1 knockdown, we decided to extensively examine the dependence on p53 in the CDK2AP1 knockdown HDFs. We observed that when p53 and CDK2AP1 were simultaneously downregulated in primary HDFs, the cells did not enter senescence based on reduced senescence associated  $\beta$ -galactosidase activity. The simultaneous knockdown of p53 and CDK2AP1 also led to reduction of accumulation of cells in the G1 phase and increased cells in the S phase of the cell cycle. qPCR analysis further demonstrated that knockdown of p53 in HDFs prevents the CDK2AP1 knockdown induced increase in *p21*, *BAX* or *PUMA* gene expression. Together, these results lead us to conclude that the CDK2AP1 knockdown induced senescence in primary HDFs is p53 dependent.

## CHAPTER 3

### **Knockdown of CDK2AP1 in Human Embryonic Stem Cells Reduces the Threshold of Differentiation**

#### **Abstract**

Recent studies have suggested a role for the Cyclin Dependent Kinase-2 Associated Protein 1 (CDK2AP1) in stem cell differentiation and self-renewal. In studies with mouse embryonic stem cells (mESCs) derived from generated mice embryos with targeted deletion of the *Cdk2ap1* gene, CDK2AP1 was shown to be required for epigenetic silencing of *Oct4* during differentiation, with deletion resulting in persistent self-renewal and reduced differentiation potential. Differentiation capacity was restored in these cells following the introduction of a non-phosphorylatable form of the retinoblastoma protein (pRb) or exogenous *Cdk2ap1*. In this study, we investigated the role of CDK2AP1 in human embryonic stem cells (hESCs). Using a shRNA to reduce its expression in hESCs, we found that CDK2AP1 knockdown resulted in a significant reduction in the expression of the pluripotency genes, *OCT4* and *NANOG*. We also found that

CDK2AP1 knockdown increased the number of embryoid bodies (EBs) formed when differentiation was induced. In addition, the generated EBs had significantly higher expression of markers of all three germ layers, indicating that CDK2AP1 knockdown enhanced differentiation. CDK2AP1 knockdown also resulted in reduced proliferation and reduced the percentage of cells in the S phase and increased cells in the G2/M phase of the cell cycle. Further investigation revealed that a higher level of p53 protein was present in the CDK2AP1 knockdown hESCs. In hESCs in which p53 and CDK2AP1 were simultaneously downregulated, *OCT4* and *NANOG* expression was not affected and percentage of cells in the S phase of the cell cycle was not reduced. Taken together, our results indicate that the knockdown of CDK2AP1 in hESCs results in increased p53 and enhances differentiation and favors it over a self-renewal fate.

## **Introduction**

Human CDK2AP1 is a highly conserved, ubiquitously expressed gene located on chromosome 12q24 and is a 115-aa nuclear polypeptide (Tsuji, Duh et al. 1998). The murine homolog of CDK2AP1 is located on chromosome number 5 and has only 3 amino acids deviations from the human protein (Kim, Ohyama et al. 2005). CDK2AP1 is also called Deleted in Oral Cancer-1 (Doc1) as it was found to be downregulated in about 70% of oral cancers (Shintani, Mihara et al. 2001). In vitro studies have revealed that CDK2AP1 mainly functions by regulating the S phase of the cell cycle, with studies showing that binding of CDK2AP1 to the DNA polymerase alpha/primase complex specifically prevents the initiation step of the DNA replication process (Matsuo, Shintani et al. 2000). It has also been shown to regulate G1/S phase progression by inhibiting CDK2 and targeting it for proteolysis (Shintani, Ohyama et al. 2000).

CDK2AP1 has lately gained importance in the field of stem cell research, with initial studies identifying it as one of the stem cell-specific genes that are enriched in both embryonic and adult stem cells (Ramalho-Santos, Yoon et al. 2002). It has also been identified as one of many genes that are expressed in early stage preimplantation embryos (Sharov, Piao et al. 2003). In studies conducted with homozygous *Cdk2ap1* knockout mESCs, the effects of LIF (leukemia Inhibitory Factor) removal on the *Cdk2ap1* knockout and wild type cells were examined (Deshpande, Dai et al. 2009). Upon the removal of LIF, *Cdk2ap1*<sup>+/+</sup> mESCs showed signs of differentiation and reduced the expression of the pluripotency gene *Oct4*, whereas the *Cdk2ap1*<sup>-/-</sup> mESCs maintained their expression of *Oct4* and did not display any signs of differentiation (Deshpande, Dai et al. 2009). Further investigation revealed that deletion of *Cdk2ap1* in mESCs prevented methylation of *Oct4* promoter which resulted in the maintenance of its expression even in 8 day old embryoid bodies. In a subsequent study, deletion of *Cdk2ap1* in mESCs prevented differentiation and resulted in persistent self-renewal, attributed to hyperphosphorylation of the retinoblastoma protein (pRb). Differentiation capacity in the *Cdk2ap1*<sup>-/-</sup> mESCs was restored upon the introduction of a mutant non-phosphorylatable form of pRb or exogenous *Cdk2ap1* (Kim, Deshpande et al. 2009). Taken together, these studies suggest that deletion of *Cdk2ap1* in mESCs results in increased self-renewal and a decrease in differentiation.

In this study, we have uncovered a novel function for CDK2AP1 in the self-renewal and pluripotency of human embryonic stem cells. Knockdown of CDK2AP1 in hESCs most likely results in increased p53 and enhances differentiation potential and favors it over self-renewal.

## **Materials and Methods**

### **Generation of Inactivated Feeder Layers and Conditioned Medium**

Mouse embryonic fibroblasts (MEFs) were isolated from embryos derived from 13.5 days pregnant CF1 mice and maintained in MEF-medium containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. MEF-conditioned medium (CM) was generated by seeding  $1 \times 10^6$  mitotically inactivated MEFs on 10 cm dishes in 10 ml MEF- medium. The following day, MEF-medium was replaced with 10 ml of human embryonic stem cell (hESC)medium containing DMEM/F-12, 20% knockout serum, 2 mM L-glutamine, 1% nonessential amino acids, 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin, 0.1 mM beta-mercaptoethanol MEF-CM medium was collected and replaced with fresh medium every day, with collected medium filtered and either used immediately to propagate hESCs or stored at  $-20^{\circ}\text{C}$ . 4ng/ml bFGF was added to MEF-CM before use for propagating hESCs. All reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise noted.

### **Propagation of Human Pluripotent Stem Cells**

Karyotypically normal diploid hESC (H9, <http://stemcells.nih.gov>) and a hiPSC line (WiCell Research Institute, Madison, WI) were routinely passaged on MEFs in 35-mm dishes. A rapidly dividing, karyotypically aneuploid cell line BG01v (ATCC, VA) hESC was also routinely passaged on MEFs in 35-mm dishes. H9 and BG01v hESCs were passaged as colonies by enzymatically methods every 3–4 days at subculturing ratios of 1:4 and maintained in hESC-medium. For use in our experiments, both H9 and BG01v hESCs were transferred onto Matrigel<sup>TM</sup> (BD Biosciences, CA) coated cell culture plates and propagated in MEF-CM.

### **Generation of hESCs Expressing CDK2AP1-Specific shRNA and p53-Specific shRNA**

We have identified two potent shRNAs targeted to CDK2AP1 mRNA. Multiple shRNAs were obtained from commercially available sources (Open Biosystems, PA; Sigma-Aldrich, MO) and screened for their effectiveness. Control scrambled sequences were used similarly. To identify the shRNA clone that produced the strongest knockdown of CDK2AP1, hESCs were transduced with the different shRNA clones using lentiviral vectors and successfully transduced cells were selected by puromycin treatment (1 µg/ml). Following 6 days of selection, antibiotic-resistant colonies were harvested and RNA extracted. QPCR analyses using human CDK2AP1 specific primers were conducted. In our experiments, one shRNA (labeled as shRNA1 henceforth) (Open Biosystems, PA) produced the strongest knockdown and was used in subsequent experiments. For rescue experiments, validated CDK2AP1 shRNA (labeled as shRNA2 henceforth) (Sigma-Aldrich, MO) that target the 3'-UTR was used. In experiments involving analysis of the role of p53, expression was downregulated using lentiviral delivery of p53-specific shRNA (Addgene, MA, USA), followed by validation of knockdown by qPCR analyses.

### **In vitro Differentiation of hESCs and Histopathology of hESC-derived Embryoid Bodies**

To generate embryoid bodies (EBs), hESCs were dissociated using collagenase and resuspended in growth medium devoid of bFGF. EB formation was facilitated using suspension culture by a hanging drop method, where cells at a density of 25,000 cells/mL were suspended from a Petri dish lid in 20 µL droplets. After 2 days, the EBs were transferred to agarose plates at a density of 25–30 EBs/10 mL to facilitate further differentiation with media changes every 3–4 days, for a total differentiation duration of 18 days. EBs were prepared for histopathological analysis by fixation in 3.7% PFA in 1.5ml microfuge tubes at approximately 15-25 EBs per tube. Once fixed overnight, EBs were rinsed with PBS to remove PFA, resuspended in 200 µl melted



4% low melting point agarose (Sigma Aldrich, MO, USA) at 42°C and incubated for 2 hours to allow settling. Final pelleting and agarose solidification was performed with brief room temperature centrifugation at 500g. Agarose-embedded samples were processed for paraffin sectioning in a Leica TP1020 tissue processor. Hematoxylin and eosin (H&E) staining was performed on microscope slide-mounted 5µm sections in a Leica Autostainer XL workstation. Images were acquired using a Nikon TS-100 microscope using the default imaging parameters. H&E staining and histopathological analysis was conducted in collaboration with Dr. Steven Sheridan at Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School.

### **RNA Isolation, Real Time Reverse Transcription Polymerase Chain Reaction, and Gene Expression Analysis**

RNA was isolated from hESCs and EBs under different conditions using RNeasy kit (Qiagen, CA, USA), according to the manufacturer's protocols and quantified using BioMate3 UV-VIS Spectrophotometer (Thermo Scientific, MA, USA). cDNA was synthesized from 1 µg of RNA using cDNA reverse transcription kit (Applied Biosystems, CA). Gene expression within different samples was analyzed using quantitative real time RT-PCR (QPCR). QPCR was performed in an ABI HT7900 system (Applied Biosystems, CA) and the data were acquired using sequence detection system software (SDS v2.2.1, Applied Biosystems, CA). Gene expression data (three replicates) were acquired and SDS software was used to estimate differential gene expression using  $\Delta$ CT quantification methods. Endogenous GAPDH was used for normalization. Commercially available primers for *CDK2AP1* and *OCT4* were obtained from

Origene, MD, while other primers summarized in Table 1 were obtained from Integrated DNA Technologies, IA.

### **Antibodies and Immunocytochemical Analysis**

Under different experimental conditions, hESCs were seeded onto four chambered glass slides. Paraformaldehyde (PFA, 4%) in PBS was used for fixation, permeabilization for intracellular markers was achieved with 0.2% Triton X-100 in PBS and blocked with normal goat serum. Fixed cells were incubated with primary antibodies: CDK2AP1 (Santa Cruz, CA, USA) and Anti-Phospho-Histone-3 (Cell Signaling, MA, USA). Goat anti-rabbit IgG conjugated to Alexa 594 (Invitrogen, CA,) was used as a secondary antibody. Fluorescent images were acquired using a Cool- Snap EZ camera (Photometrics, Tucson, AZ) mounted on a Nikon Eclipse TE 2000-S inverted microscope (Nikon, Melville, NY) with attached image analysis software. All image settings were controlled for uniform acquisition between samples. Specifically, uniform exposure time was maintained for images acquired from experimental samples as well as negative controls for background subtraction. For experiments involving determination of mitotic index based on phospho-histone 3 staining, random fields were selected and counting was performed in triplicate by counting around 500 cells during each analyses. Mitotic index was calculated by dividing the number of phospho-histone 3 positive cells, over the total number of cells.

**Table 2: Sequences of Forward and Reverse Primers Used in qPCR Analysis.**

Primer	Sequence
GAPDH Forward	TTGCCATCAATGACCCCTTCA
GAPDH Reverse	CGCCCCACTTGATTTTGGGA
CDK2AP1 Forward	ATGTCTTACAAACCGAACTTGGC
CDK2AP1 Reverse	GCCCGTAGTCACTGAGCAG
SOX1 Forward	ATGCACCGCTACGACATGG
SOX1 Reverse	CTCATGTAGCCCTGCGAGTTG
NESTIN Forward	CTGCTACCCTTGAGACACCTG
NESTIN Reverse	GGGCTCTGATCTCTGCATCTAC
T Brachyury Forward	TGCTTCCCTGAGACCCAGTT
T Brachyury Reverse	GATCACTTCTTTCCTTTGCATCAAG
IGF2 Forward	TCCTCCCTGGACAATCAGAC
IGF2 Reverse	AGAAGCACCAGCATCGACTT
AFP Forward	CTTTGGGCTGCTCGCTATGA
AFP Reverse	GCATGTTGATTTAACAAGCTGCT
GATA4 Forward	GTGTCCCAGACGTTCTCAGTC
GATA4 Reverse	GGGAGACGCATAGCCTTGT
CDKN1A (p21) Forward	TGTCCGTCAGAACCCATGC
CDKN1A (p21) Reverse	AAAGTCGAAGTTCCATCGCTC
Tp53 Forward	CAGCACATGACGGAGGTTGT
Tp53 Reverse	TCATCCAAATACTCCACACGC

## **Western Blot Analysis**

Cells for analysis were harvested by trypsinization, centrifuged at 1000 rpm and washed once with ice-cold PBS buffer. Cells were then lysed using the Total Protein Extraction Kit (EMD Millipore, MA)) based on protocol provided by the manufacturer. Cell lysates were then subjected to Western blot analyses using specific antibodies to various cyclins. Cell lysates were prepared from wild type and CDK2AP1 knockdown H9 hESCs and analyzed for CDK2AP1, OCT4, NANOG, p53, Cyclin A1 expression by Western blot using specific antibodies. The CDK2AP1, OCT4, NANOG and Cyclin A1 antibodies were obtained from Santa Cruz, CA, USA, while p53 antibody was obtained from Cell Signaling, MA, USA. Appropriate infrared emitting-conjugated secondary antibodies were obtained from Invitrogen, CA. Detection was then carried out using the Odyssey Infrared Imaging System (Li-Cor Biosciences, NE). The quantitative analysis of the Western Blot bands was carried out using the ImageJ software (Schneider, Rasband et al. 2012).

## **Cell Cycle Profile Analysis**

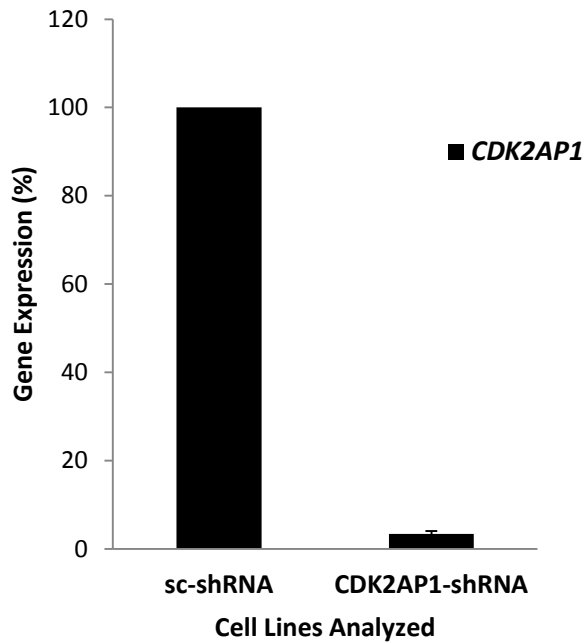
Cells to be analyzed were trypsinized, washed, stained with propidium iodide for 45 min at 37°C, filtered through a 30 µm mesh to eliminate clumps and subjected to cell cycle analysis on a Accuri™ C6 Flow Cytometer (BD Biosciences, CA). Data were analyzed using the software provided by the manufacturer and samples analyzed in triplicate.

## Results

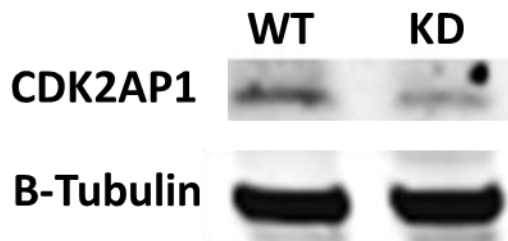
### Knockdown of CDK2AP1 in hESCs Reduces *OCT4* and *NANOG* Expression

To investigate the effect of CDK2AP1 knockdown on the expression of the core pluripotent genes *OCT4* and *NANOG*, we used lentiviral delivery of shRNA1 to downregulate CDK2AP1 in H9 hESC line. The CDK2AP1 shRNA clones tested utilize the U6 promoter to drive the expression of the shRNA's, with the U6 promoter shown to be very active in undifferentiated hESCs (Xia, Zhang 2007). Following the first passage after transduction, antibiotic-resistant colonies were selected upon exposure to puromycin at a concentration of 1µg/ml for 7 days. CDK2AP1 expression was then examined using qPCR (Figure 16.A), and Western blot (Figure 16.B). Based on qPCR analysis, we were able to achieve 96% knockdown of CDK2AP1, with statistical analysis indicating significant differences ( $p < 0.05$ ) in the expression patterns between hESCs transduced with the shRNA1 and the scshRNA (Figure 16.A). We also noted that the knockdown of CDK2AP1 resulted in a statistically significant reduction ( $p < 0.05$ ) in *OCT4* and *NANOG* levels by 63.3% and 66.1% respectively (Figure 17). In addition, when a second CDK2AP1 specific shRNA was used we were able to achieve 98% knockdown which also resulted in a reduction ( $p < 0.05$ ) in *OCT4* and *NANOG* levels by 72.9% and 53% respectively. This data suggests a role for CDK2AP1 in affecting pluripotent gene expression of hESCs.

**A.**

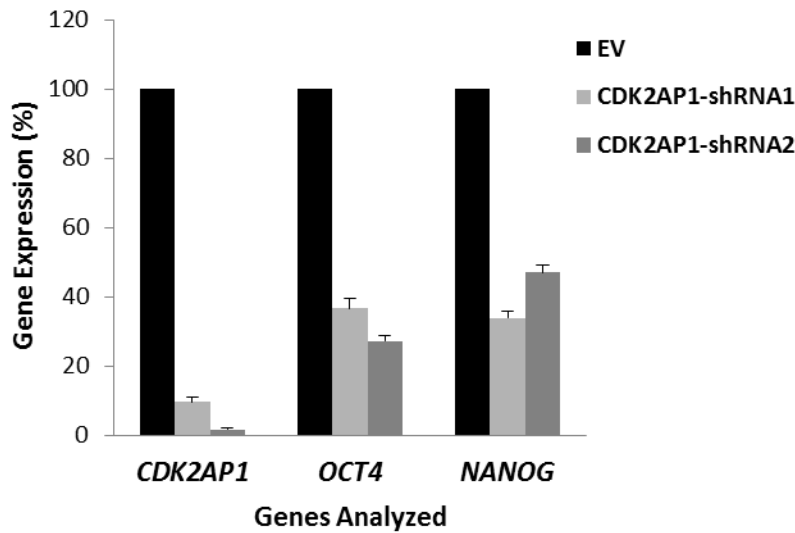


**B.**

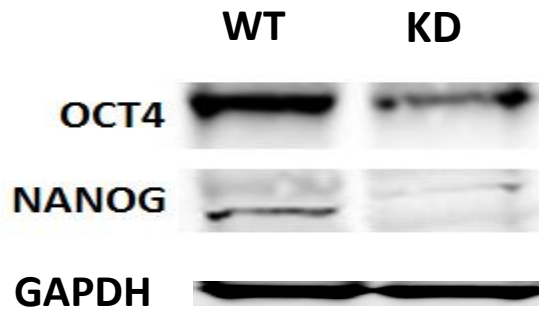


**Figure 16: CDK2AP1 is Successfully Downregulated in hESCs.** **A.** H9 hESCs were transduced with a sc-shRNA or CDK2AP1-shRNA1. After 7 days of puromycin selection at 1  $\mu\text{g/ml}$ , total RNA was extracted and real time-qPCR was used to measure the expression of CDK2AP1. The cells that were transduced with CDK2AP1-shRNA1 had 96% reduction in CDK2AP1 expression (p-value <0.05). Results are presented together with standard deviation from experiments conducted in triplicate. **B.** Whole cell lysates were used to examine levels of CDK2AP1 protein in H9 hESCs transduced with CDK2AP1 specific shRNA1 (right lane) or a control (left). Cells that were transduced with CDK2AP1 specific shRNA1 had lower CDK2AP1 protein levels.

A.



B.



**Figure 17: Knockdown of CDK2AP1 in hESCs Reduces Levels of OCT4 and NANOG.** **A.** H9 hESCs that were transduced with CDK2AP1-shRNA1, CDK2AP1-shRNA2 or an empty vector (EV) were examined for *CDK2AP1* knockdown and for *OCT4* and *NANOG* expression. Knockdown of CDK2AP1 using CDK2AP1-shRNA1 and CDK2AP1-shRNA2 resulted in reduction in *OCT4* and *NANOG* expression when compared with hESCs that were transduced with the empty vector (p-value < 0.05. Comparisons were made between EV and CDK2AP1-shRNA1 or EV and CDK2AP1-shRNA2 transduced cells). Results are presented together with standard deviation from experiments conducted in triplicate. **B.** Whole cell lysates from H9s transduced with CDK2AP1-shRNA1 (right lane) or empty vector (left) were examined by Western Analysis and demonstrated reduction in OCT4 and NANOG levels.

### **Knockdown of CDK2AP1 Enhances EB Formation**

Previous studies that were performed on mESCs showed that *Cdk2ap1* knockout mESCs were resistant to differentiation and yielded lower number of embryoid bodies (EB) in which *Oct4* was still active (Deshpande, Dai et al. 2009). These studies demonstrated the inability to silence *Oct4* expression in 8-day old EBs derived from *Cdk2ap1* knockout mESCs. We thus examined the effect of CDK2AP1 knockdown on EB formation and *Oct4* gene expression in these EBs derived from hESCs. Using the hanging drop method, we generated EBs from H9 hESCs transduced with either a scrambled shRNA or CDK2AP1-shRNA1. Our results indicate that a statistically significantly higher number of EBs ( $p < 0.05$ ) were generated from the knockdown when compared to wild-type cells, with parallel experiments conducted with human iPSCs yielding similar results (Figure 18). In addition, we observed a size difference between the EBs generated from wild type and knockdown hESCs, with the EBs generated from CDK2AP1 knockdown hESCs larger in size (Figure 18). Interestingly, this outcome suggests a potentially different role for CDK2AP1 in human pluripotent stem cells as the downregulation of this protein in mESCs resulted in a reduction in the number of EBs obtained. In addition, the knockdown of CDK2AP1 in human pluripotent stem cells enhanced their differentiation capability, yielding a significantly higher number of EBs which could be used in subsequent steps that could yield multiple terminally differentiated cell types.

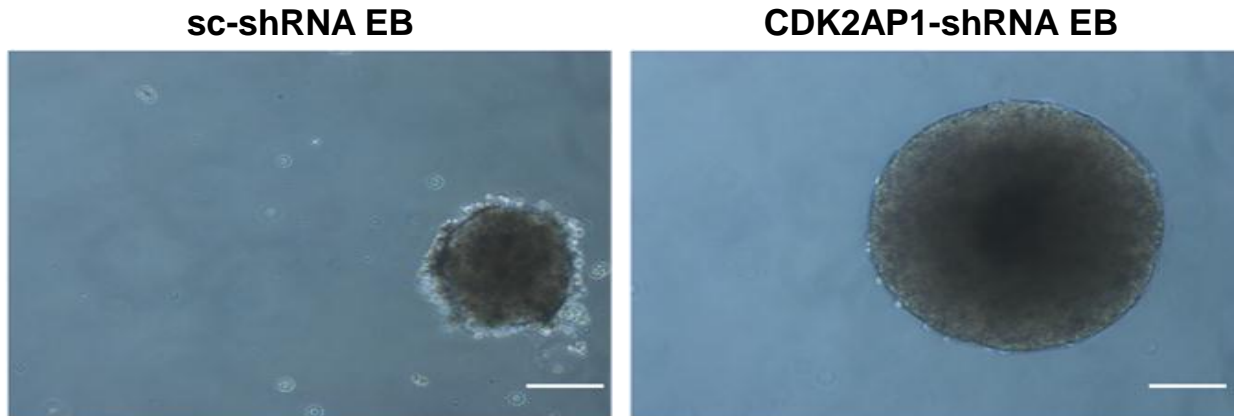
### **Knockdown of CDK2AP1 in hESCs Results in Increased In Vitro Differentiation Potential.**

A hallmark of differentiation in EBs is the formation of the three germ layers (ectoderm, mesoderm, endoderm). We next examined the expression of germ layer-specific markers in the EBs generated from CDK2AP1 knockdown H9 hESCs by qPCR analysis. EBs generated from

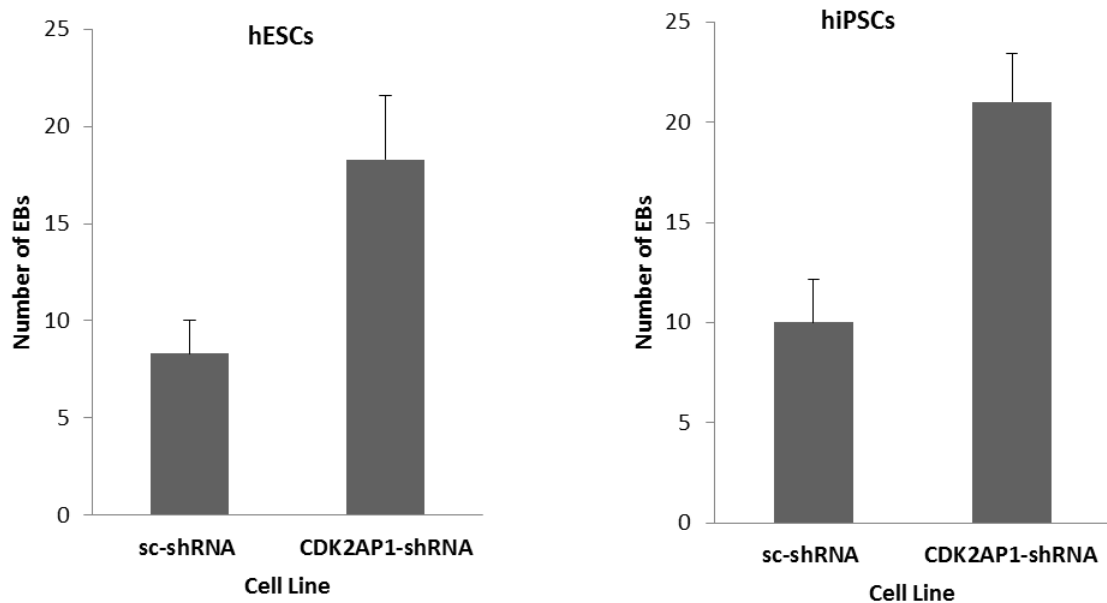


CDK2AP1 knockdown hESCs had a statistically significantly higher expression ( $p < 0.05$ ) of markers of all the three germ layers, when compared with EBs obtained from wild type hESCs (Table 3). This was specifically apparent in the expression of the mesoderm marker *T* (increased 27.6 fold) and in the ectoderm marker, *SOX1* (increased by 67 fold). We also conducted histopathological analysis of the EBs generated from CDK2AP1 knockdown H9-hESCs. When compared with wild type EBs which consisted almost exclusively of neuroectoderm, EBs generated from CDK2AP1 knockdown H9-hESCs exhibited evidence of ectoderm and mesoderm differentiation (Figure 19). Taken together, these data demonstrated that the knockdown of CDK2AP1 enhanced the differentiation of hESCs and increased the expression of markers of all three germ layers in the derived EBs. To test whether CDK2AP1 is required for *OCT4* silencing in the generated EBs, we collected 8-day old EBs generated from sc-shRNA H9 hESCs and CDK2AP1-shRNA H9 hESCs and measured the levels of *OCT4* expression using qPCR analysis. Our results indicate that *OCT4* was equally silenced in both CDK2AP1 knockdown and wild type EBs (Figure 20), thus indicating that CDK2AP1 is not required for *OCT4* silencing during the differentiation of hESCs.

A.



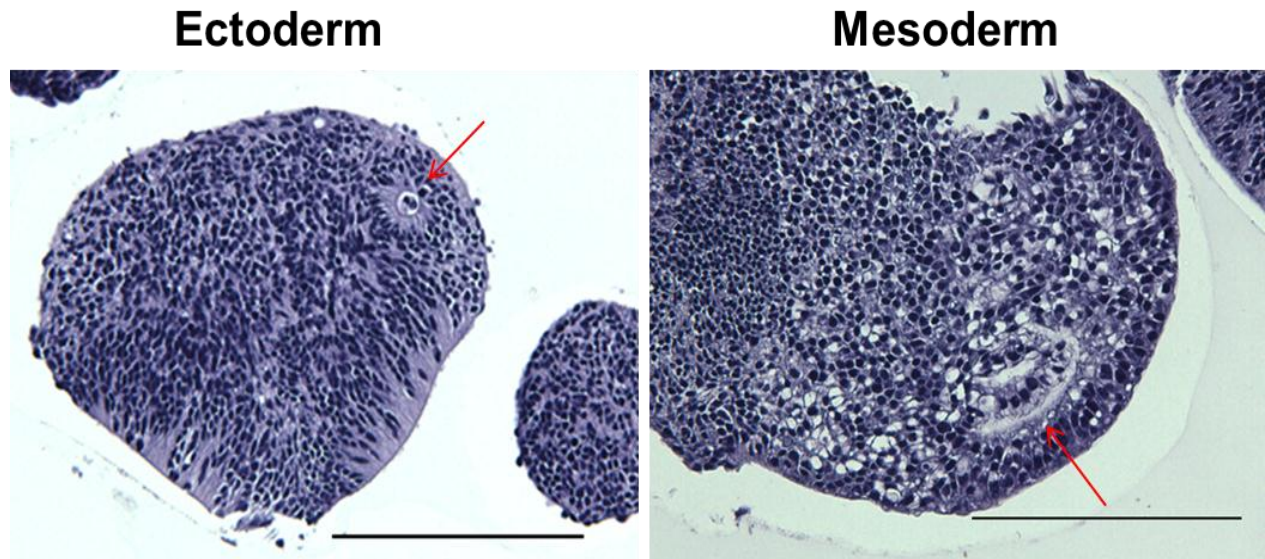
B.



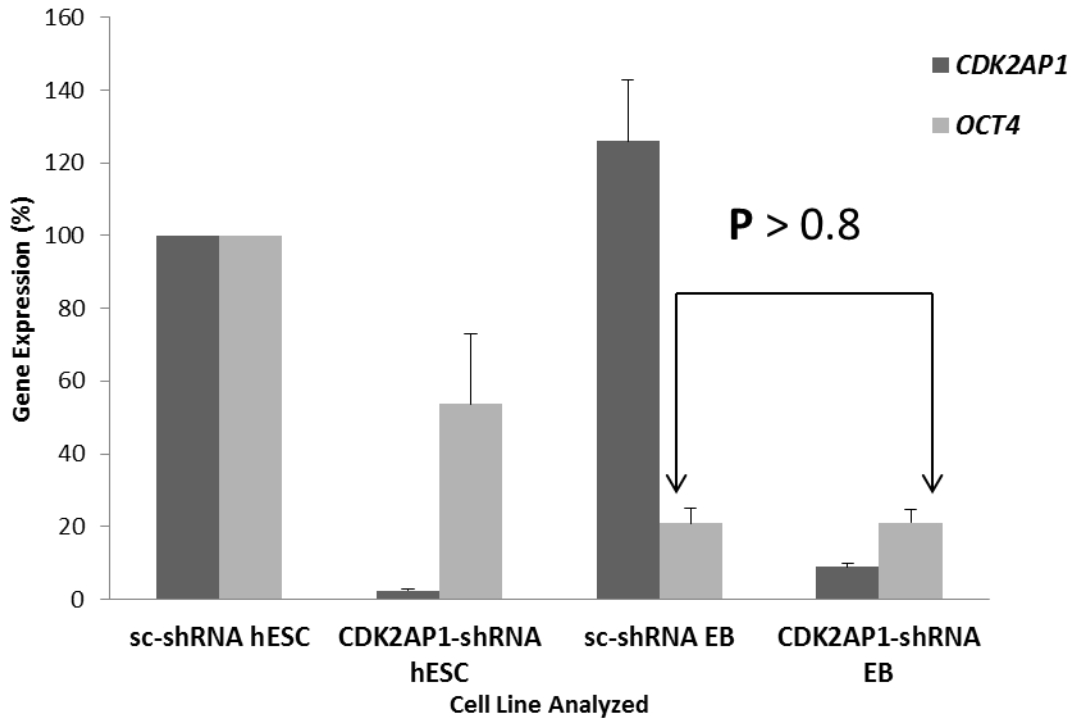
**Figure 18: Knockdown of CDK2AP1 in hESCs Increases the Size and Number of Generated EBs.** A. Representative EBs that were generated from wild-type and CDK2AP1-knockdown H9- hESCs shown at the same magnification. Scale bar represents 100 μm. EBs generated from CDK2AP1 knockdown hESCs appeared larger in size. B. Knockdown of CDK2AP1 in hESC (left) and hiPSCs (right) increased the number of generated EBs ( $p < 0.05$ ). Results are presented together with standard deviation from experiments conducted in triplicate.

**Table 3: Knockdown of CDK2AP1 in H9-hESCs Results in EBs with Enhanced Expression of Genes Corresponding to the Three Germ Layers.** Quantitative PCR data showing the effect of CDK2AP1 knockdown on the expression of the germ layers markers in EBs derived from H9 hESCs. EBs obtained from sc-shRNA transduced H9s and EBs obtained from CDK2AP1-shRNA were harvested and the expression of markers of germ layers (Endoderm: *AFP* and *GATA4*. Mesoderm: *T* and *IGF2*. Ectoderm: *SOX1* and *NESTIN*) was examined by qPCR. Knockdown of CDK2AP1 significantly enhanced the expression of all markers of differentiation (p-value < 0.05).

Germ Layer Marker		Fold Change	p-value
Endoderm Markers	<i>AFP</i>	1.9 ↑	0.01
	<i>GATA4</i>	2.1 ↑	2.0E-04
Mesoderm Markers	<i>T</i>	27.6 ↑	1.9E-06
	<i>IGF2</i>	4.8 ↑	4.8E-05
Ectoderm Markers	<i>SOX1</i>	67.2 ↑	1.2E-06
	<i>NESTIN</i>	3.5 ↑	0.002



**Figure 19: Knockdown of CDK2AP1 Enhances Differentiation.** Shown are images of hematoxylin and eosin-stained histopathologic sections of EBs generated from CDK2AP1 knockdown H9 hESCs. Representative differentiation is shown based (a) ectodermal (neuroepithelial) and (b) mesodermal (fibrous connective) germ layer formation. Scale bar represents 100 μm.



**Figure 20: CDK2AP1 is not Required for *OCT4* Silencing During the Differentiation of hESCs.** To investigate if CDK2AP1 is required for proper silencing of *OCT4* during hESC, 8-day old EBs generated from CDK2AP1 wild type (sc-shRNA) and knockdown (CDK2AP1-shRNA) hESCs were harvested and the levels of *CDK2AP1* and *OCT4* measured by qPCR. Results indicate that CDK2AP1 knockdown hESCs were able to shut down *OCT4* expression to the same levels seen in the wild type EBs (p-value = 0.88). Results are presented together with standard deviation from experiments conducted in triplicate.

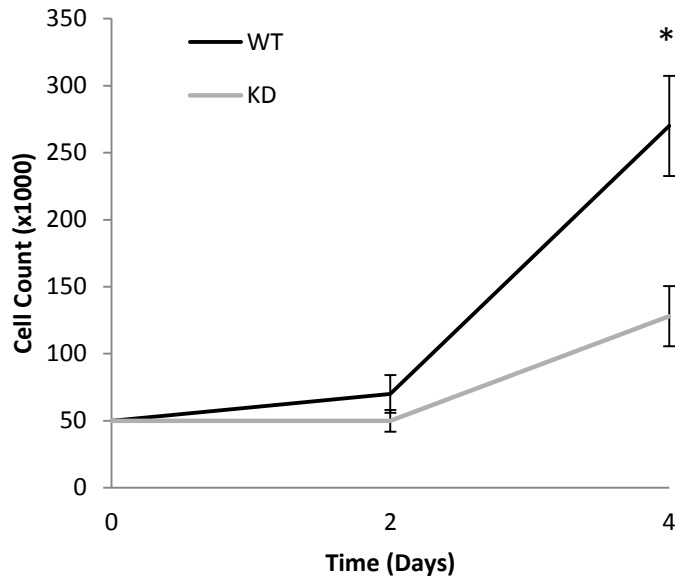
### **Knockdown of CDK2AP1 in hESCs Reduces Proliferation and Alters Cell Cycle Profile**

Downregulation of *Cdk2ap1* in mESCs resulted in an increase in proliferation with hypersphosphorylation of pRb (Kim, Deshpande et al. 2009). The differentiation capacity of these mESCs was extremely reduced and was only restored following the introduction of exogenous *Cdk2ap1*, or by expressing a mutated form of *pRb* that was resistant to phosphorylation (Kim, Deshpande et al. 2009). This study presented a clear manifestation of the strong relationship between cell cycle regulation, pluripotency and differentiation. Being an inhibitor of CDK2, we initially expected that knockdown of CDK2AP1 would increase proliferation of hESCs and also reduce their differentiation capacity by pushing them more towards self-renewal. However, as mentioned above, we found that the knockdown of CDK2AP1 enhanced differentiation potential of the hESCs. We thus decided to examine the effect of the knockdown on the proliferation and cell cycle profile of hESCs.

Fifty thousand H9 hESCs that were transduced with either an empty vector or with a CDK2AP1 specific shRNA1 were seeded per well in a 12-well plate and were counted at 2 and 4 days post seeding. We observed that the knockdown of CDK2AP1 significantly reduced the proliferation of hESCs ( $p < 0.05$ ) (Figure 21). Cell cycle analysis demonstrated that the knockdown of CDK2AP1 significantly decreased cells in the S phase from 23% in wild type cells to an average of 14.6% in CDK2AP1 shRNA1 transduced cells ( $p\text{-value} = 0.0007$ ). In addition, knockdown resulted in a significant increase in the percentage of cells in the G2/M ( $p\text{-value} = 0.0008$ ) when compared with shRNA1 transduced cells from 36.7% to 45.6% (Figure 22).

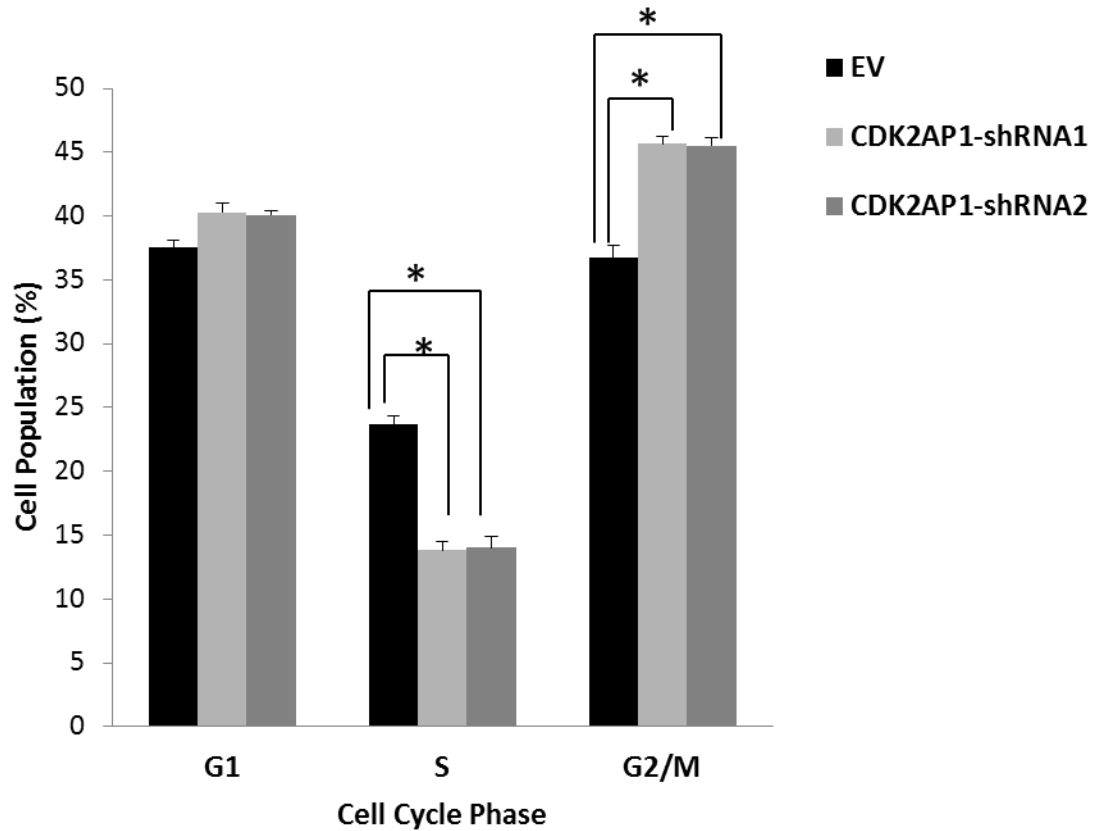
After we observed the significant increase in the percentage of CDK2AP1 knockdown hESCs that were in the G2/M phase of the cell cycle, we examined the protein levels of Cyclin A1 in those cells. It has been reported that increased Cyclin A1 can cause the accumulation of cells in the G2/M phase of the cell cycle, mitotic catastrophe and apoptosis (Rivera, A. et al. 2006). Whole cell lysates of H9 hESCs that were transduced with an empty vector or CDK2AP1 shRNA1 were examined for Cyclin A1 protein levels. We found that knockdown of CDK2AP1 increased the levels of Cyclin A1 by two fold (Figure 23). We also examined the levels of phospho-Histone 3 (p-H3) which is detectable in cells that are in the late G2 and M phase of cell cycle (Van Hooser, Goodrich et al. 1998). Immunocytochemical analyses indicated that a significantly higher percentage (4.7%) of CDK2AP1 knockdown H9-hESCs stained positive for p-H3, while only (2.6%) of wild-type hESCs were positive ( $p < 0.05$ ) (Figure 24).

These results indicate that CDK2AP1 may play a different role in hESCs when compared to mESCs. In *Cdk2ap1*<sup>-/-</sup> mESCs, differentiation capacity was compromised, whereas we found that CDK2AP1 knockdown H9-hESCs had a significant reduction in the expression of the pluripotency genes, *OCT4* and *NANOG*, and generated higher number of EBs which had higher expression of candidate markers of the three germ layers when they were differentiated. *Cdk2ap1*<sup>-/-</sup> mESCs seemed to favor self-renewal over differentiation, which was apparent in their faster cell cycle and the hyperphosphorylation of the pRb. On the other hand, our results indicate that CDK2AP1 knockdown H9-hESCs seem to favor differentiation over self-renewal, as evinced by their reduced proliferation and reduced pluripotency gene expression.

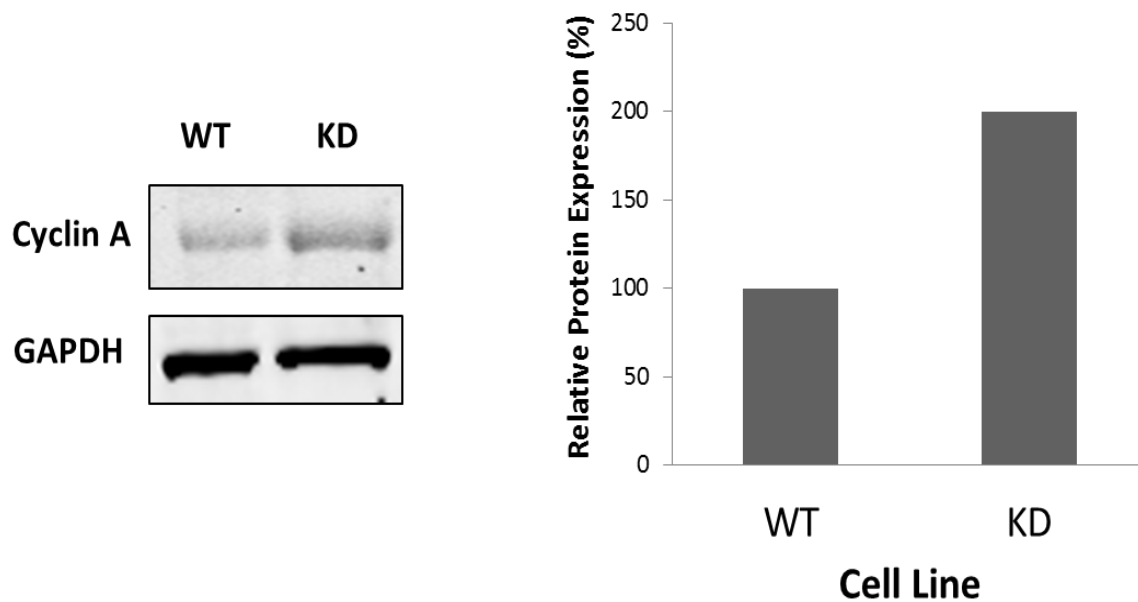


**Figure 21: Knockdown of CDK2AP1 in hESCs Reduces Proliferation.** Fifty thousand H9 hESCs that were transduced with an empty vector (WT) or CDK2AP1 shRNA1 (KD) were seeded per well in a 12-well plate. Cells were harvested and counted in triplicates at 2 and 4 days post seeding. (\*- p-value < 0.05. Comparisons were made between WT and KD cells at respective time points). Results are presented together with standard deviation from experiments conducted in triplicate.



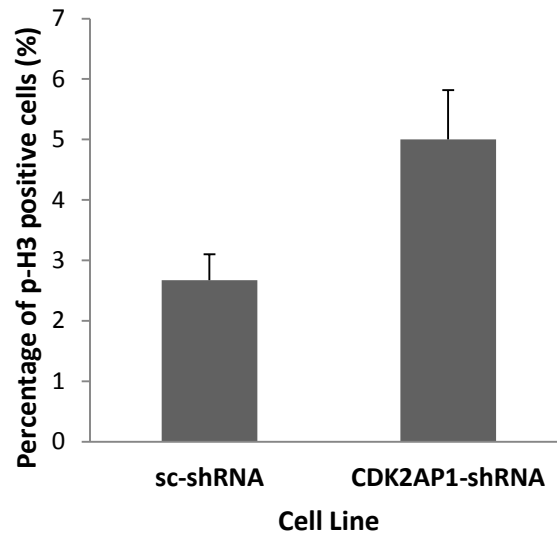


**Figure 22: Knockdown of CDK2AP1 in hESCs Increases Cells in the G2/M and Decreases Cells in the S Phase of the Cell Cycle.** CDK2AP1 wild type and knockdown H9 hESCs were harvested and equal numbers were stained with propidium iodide (PI) and analyzed using an Accuri C6 flow cytometer. Results are presented together with standard deviation from experiments conducted in triplicate. (\*=  $p < 0.05$ ).

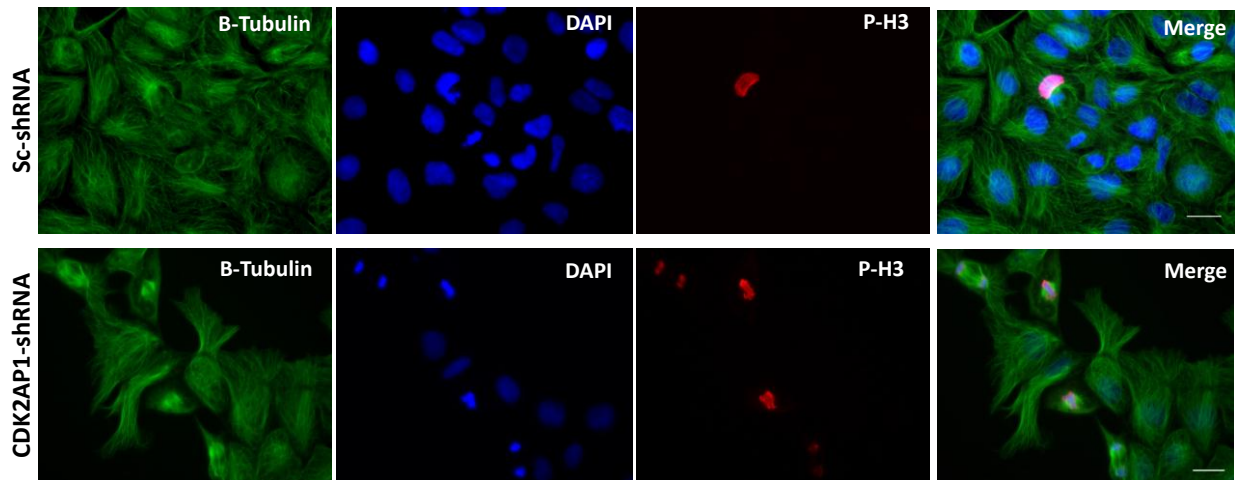


**Figure 23: Knockdown of CDK2AP1 in hESCs Increases Cyclin A levels.** Western blot (left) shows an increase in Cyclin A levels in the CDK2AP1 knockdown hESCs when compared to wild type cells. Densitometric analysis of the protein levels of the Western blot is presented in the right panel.

**A.**



**B.**

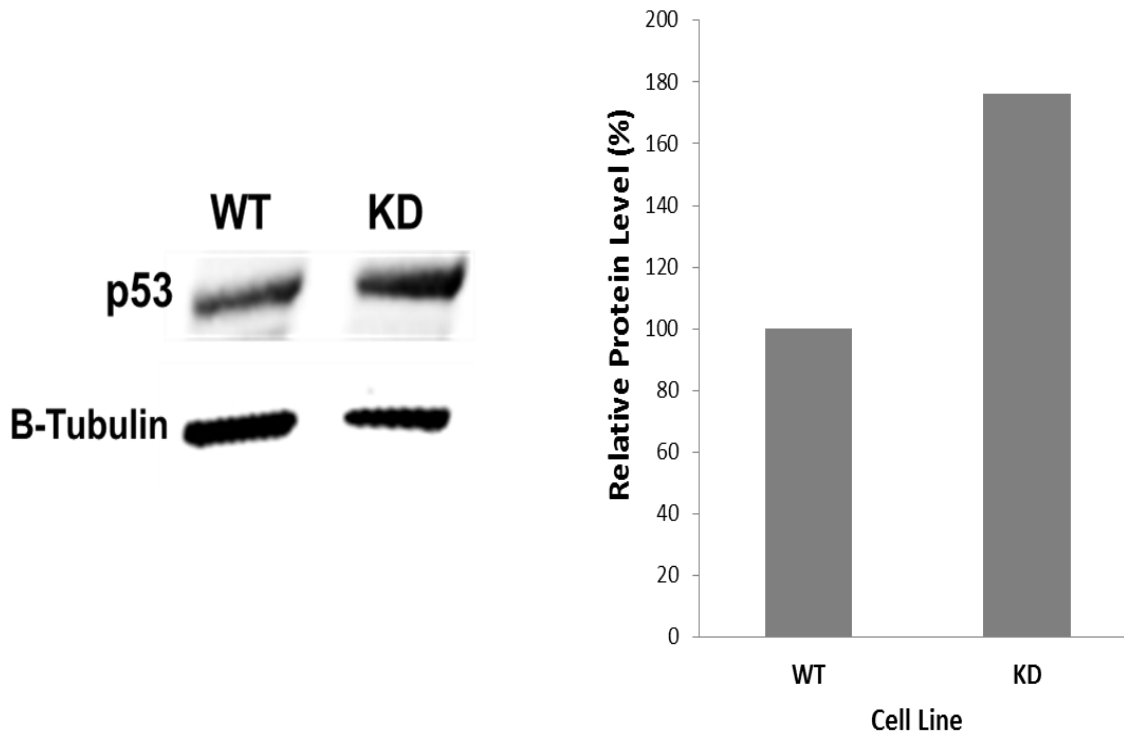


**Figure 24: Knockdown of CDK2AP1 in H9 hESC Increases the Level of Phospho-Histone 3.** H9 hESCs were transduced with a scrambled shRNA (sc-shRNA) or with CDK2AP1-shRNA1. Cells were fixed and stained using a phospho-Histone 3 specific antibody. Around 500 cells were counted in randomly selected fields and the percentage of p-H3 positive cells was calculated. A. Shows that the percentage of p-H3 positive cells. Results are presented together with standard deviation from experiments conducted in triplicate. B. Shows the p-H3 staining, DAPI,  $\beta$ -Tubulin and a merge picture in both sc-shRNA and CDK2AP1-shRNA transduced cells. Scale bar represents 50  $\mu$ m.

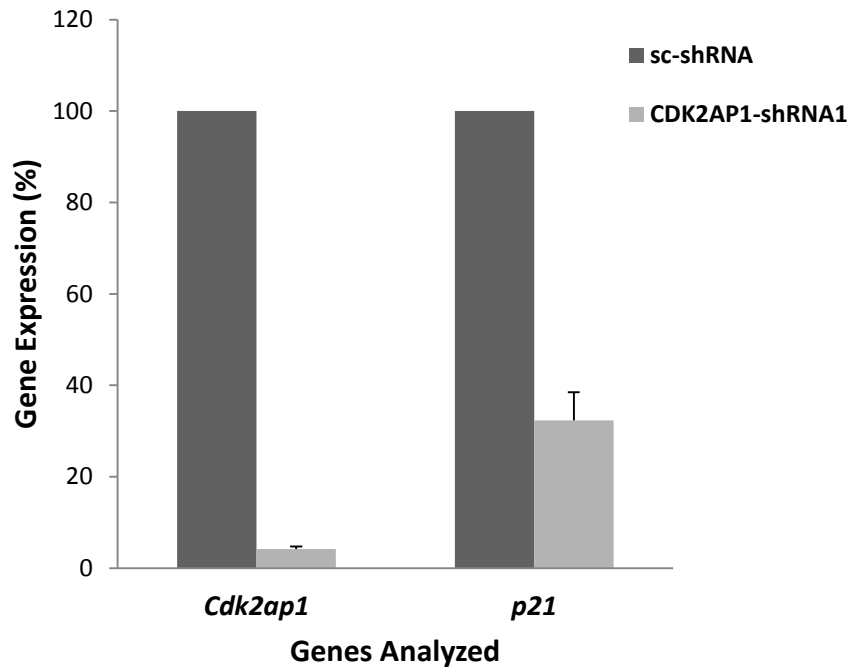
### **Knockdown of CDK2AP1 in hESCs Results in an Increase in p53 Protein Levels and Reduces *p21* mRNA Expression**

After we observed the increase in Cyclin A1 protein levels following CDK2AP1 knockdown, we decided to examine the levels of p53 in CDK2AP1 knockdown hESCs. It has been reported that increased p53 levels may increase Cyclin A1 leading to the accumulation of cells in the G2/M phase (Rivera, A. et al. 2006). In addition, we suspected that following CDK2AP1 knockdown, its inhibition on CDK2 might be alleviated leading to upregulation of the oncogene E2F, which in turn could increase Cyclin A and p53 levels (Soucek, Pusch et al. 1997). Whole cell lysate from CDK2AP1 wild type and knockdown H9 hESCs were examined for p53 protein levels by Western blot analysis. We found that knockdown of CDK2AP1 resulted in a 1.76 fold increase in p53 protein levels (Figure 25).

We next examined the expression of *p21* mRNA to test if it was affected by the increase in p53. Contrary to what was expected, qPCR analysis indicated that *p21* mRNA levels were reduced by ~60% in the CDK2AP1 knockdown H9-hESC when compared with the wild type H9-hESCs (Figure 26). It has recently been reported that in hESCs, p53 can activate the micro-RNA's: miR-302a, miR-302b, miR-302c and miR-302d, which can subsequently downregulate the *p21* mRNA when p53 is activated (Dolezalova, Mraz et al. 2012). This study clearly explained the reason behind the non-functional p53-p21 axis of the G1/S phase check point in hESCs compared to somatic cells, which activate p21 in response to p53 stabilization and arrest in the G1 phase of the cell cycle. Although we observed a decrease in *p21* expression, we did not see an increase in G1 to S phase transition. It could be that CDK2AP1 knockdown have affected the levels of other G1 checkpoint regulators like p27 or p16.



**Figure 25: Knockdown of CDK2AP1 in hESCs increases p53 levels.** Whole cell lysates of wild type and CDK2AP1 knockdown H9 hESCs were examined for p53 protein levels. We found that knockdown of CDK2AP1 increased p53 protein levels as shown by the western blot (left). Densitometric analysis of the protein level is presented in the right panel.



**Figure 26: Knockdown of CDK2AP1 in hESCs Reduces p21 Expression.** Quantitative PCR analysis showing the levels of *CDK2AP1* and *p21* expression in wild type and CDK2AP1 knockdown H9 hESCs. Knockdown of CDK2AP1 resulted in a 63% reduction in *p21* expression ( $p < 0.05$ . Comparisons were made between sc-shRNA and CDK2AP1-shRNA1 transduced cells for each gene analyzed). Results are presented together with standard deviation from experiments conducted in triplicate.

### **Simultaneous Knockdown of CDK2AP1 and p53 Prevents the Reduction in *OCT4* and *NANOG* Levels and Also Prevents the Accumulation in the G2/M Phase of the Cell Cycle.**

In a recent study, it was demonstrated that knockdown of p53 prevented retinoic acid mediated differentiation of hESCs (Jain, Allton et al. 2012). The expression of pluripotency markers, *OCT4* and *NANOG* remained elevated even after 3 days of treatment with retinoic acid in the p53 knockdown hESCs. Further investigation revealed that upon the induction of differentiation in hESCs (by retinoic acid treatment), there was an enrichment in p53 binding to p53 responsive elements in the promoters of miR-34a and miR-145 (Jain, Allton et al. 2012). MiR-34a is known to exacerbate p53 activation by inhibiting the p53-inhibitor, SIRT-1 (Yamakuchi, Ferlito et al. 2008), while miR-145 is known to suppress the expression of the pluripotency genes, *OCT4*, *SOX2*, and *KLF4* in hESCs and promote differentiation (Xu, Papagiannakopoulos et al. 2009). Taken together, we expect that the reduced expression of the pluripotency genes, *OCT4* and *NANOG* that is seen in the CDK2AP1 knockdown hESCs is due to p53 activation, which also affected the cell cycle profile and pluripotency.

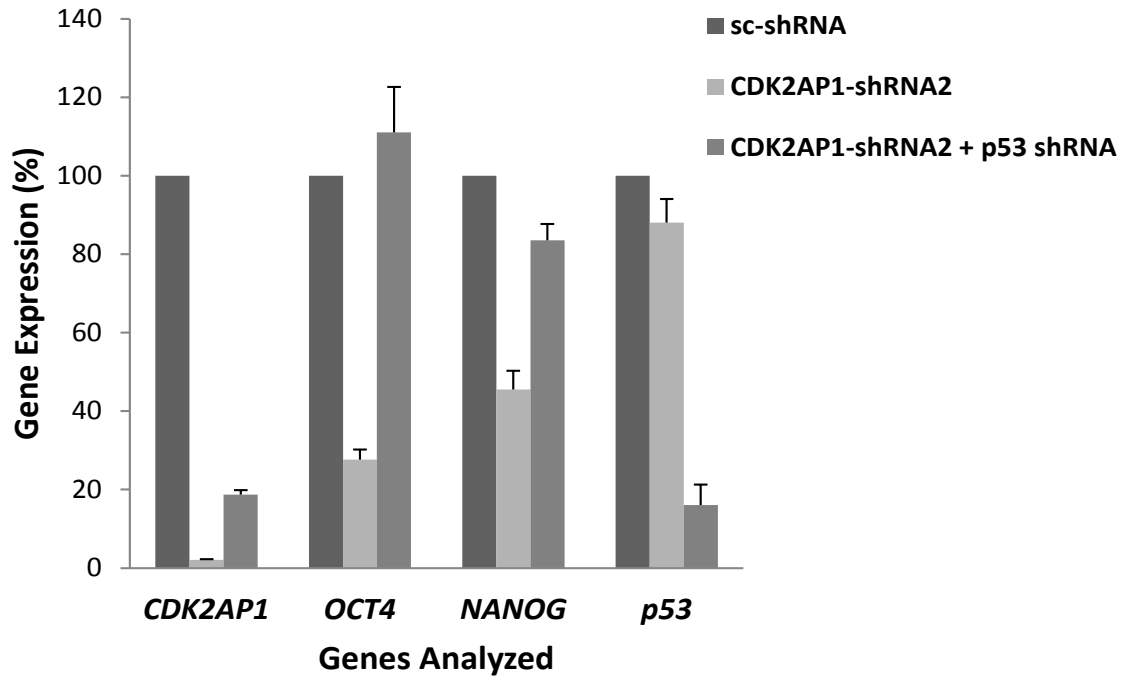
To investigate whether the observed phenotype seen in CDK2AP1 knockdown hESCs is p53 dependent, we co-transduced BG01v hESCs with p53 and CDK2AP1 shRNAs and assayed *OCT4* and *NANOG* expression by qPCR analysis. The double knockdown cells did not silence *OCT4* (p-value = 0.004) or *NANOG* (p-value= 0.0005) expression when compared with CDK2AP1 only knockdown cells (Figure 27). Similarly, introduction of exogenous *CDK2AP1* prevented the reduction in *OCT4* and *NANOG* expression seen in CDK2AP1 knockdown hESCs (Figure 28).

In experiments conducted with H9-hESCs, we found that CDK2AP1 knockdown resulted in a decrease in the percentage of cells that were in the S phase and increased cells in the G2/M phase of the cell cycle (Figure 22). To test if this shift in the cell cycle is p53 dependent, we examined the cell cycle of H9 hESCs that were co-transduced with p53 and CDK2AP1 shRNAs. We found that these cells in which p53 and CDK2AP1 were downregulated did not accumulate in the G2/M phase and had a significantly higher percentage of cells in the S phase when compared to CDK2AP1 knockdown cells (Figure 29). We noted a slight delay in S-G2/M transition in the double knockdown cells, which could be due to abnormalities in DNA synthesis as both CDK2AP1 and p53 regulate DNA replication (Zhou and Privies, 2003) (Matsuo, Shintani et al., 2000).

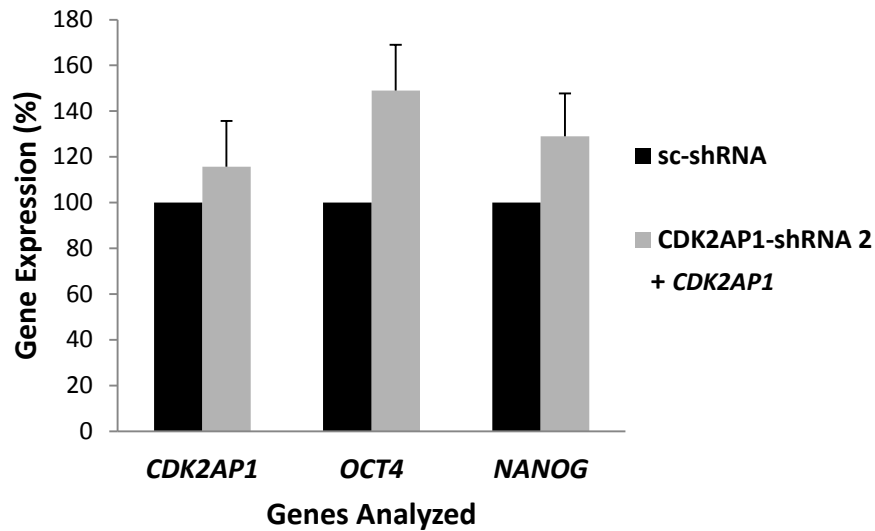
## Discussion and Conclusions

The function of CDK2AP1 as initially assessed in mESCs indicated an important role in the differentiation and self-renewal of pluripotent stem cells (Kim, Deshpande et al. 2009). Its deletion in mESCs resulted in an increase in self-renewal and a reduction in differentiation capacity, caused by a defect in the proper epigenetic silencing of *Oct4* during differentiation due to the delocalization of the Nucleosome Remodeling and Deacetylation (NuRD) complex from Oct4 promoter (Deshpande, Dai et al. 2009). Therefore, it was concluded that Cdk2ap1 is required for epigenetic silencing of Oct4 during mESC differentiation. Another study demonstrated that, *Cdk2ap1*<sup>-/-</sup> mESCs failed to differentiate due to increased phosphorylation of pRb which lead to persistent self-renewal (Kim, Deshpande et al. 2009). Differentiation capacity was restored in those cells after introduction of an unphosphorylatable form of pRb or exogenous *Cdk2ap1*.

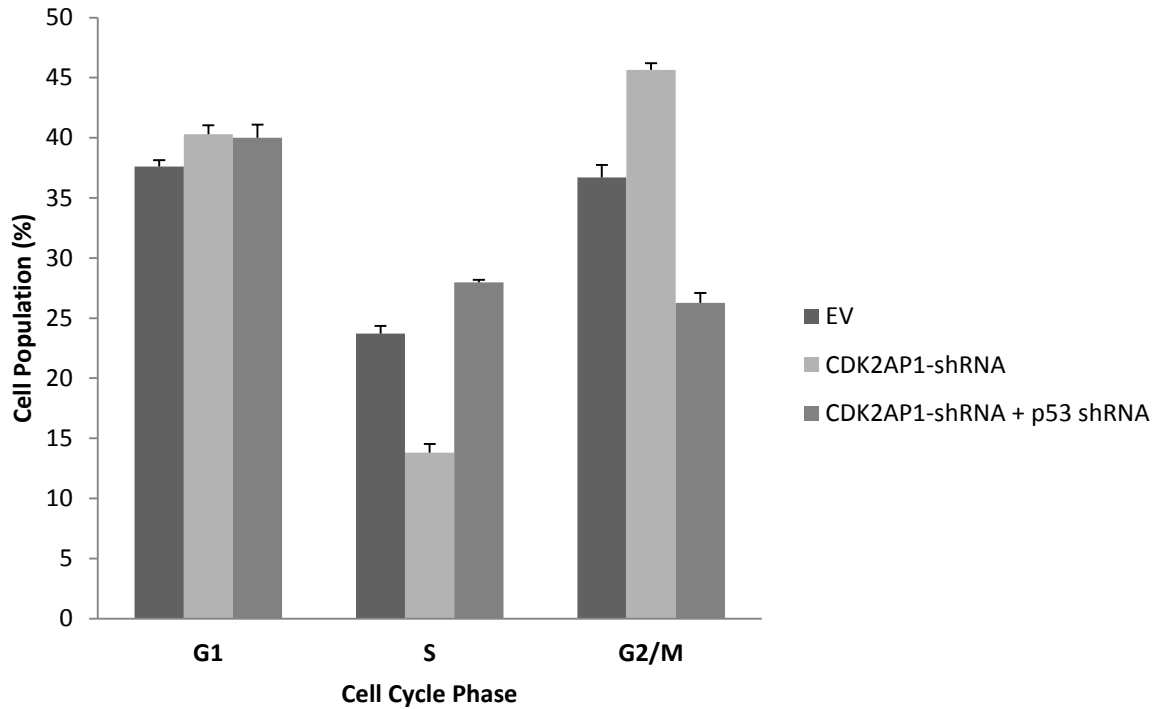




**Figure 27: Downregulation of p53 Prevents the Reduction in *OCT4* and *NANOG* Expression Seen in *CDK2AP1* only Knockdown hESCs.** BG01v hESCs were transduced with scrambled shRNA (sc-shRNA), *CDK2AP1* shRNA or co-transduced with *CDK2AP1* and p53 shRNAs. The gene expression of *CDK2AP1*, *OCT4*, *NANOG* and *p53* was analyzed in those cells by qPCR. We found that down regulating p53 with *CDK2AP1* prevented the *CDK2AP1*-knockdown induced reduction in *OCT4* and *NANOG* levels ( $p < 0.05$ . Comparisons were made between EV and *CDK2AP1*-shRNA or *CDK2AP1*-shRNA and *CDK2AP1*-shRNA + shp53 transduced cells for each gene analyzed). Results are presented together with standard deviation from experiments conducted in triplicate.



**Figure 28: Introduction of Exogenous *CDK2AP1* Simultaneously with *CDK2AP1* shRNA2 Prevents Reduction in *OCT4* and *NANOG* Expression.** BG01v hESCs were transduced with sc-shRNA or with exogenous *CDK2AP1* + *CDK2AP1* shRNA2 and analyzed by qPCR for *OCT4* and *NANOG* expression. Prevention of knockdown by introducing exogenous *CDK2AP1* prevents the reduction in *OCT4* and *NANOG* expression seen in *CDK2AP1* knockdown hESCs ( $p > 0.05$ . Comparisons were made between sc-shRNA and *CDK2AP1*-shRNA2 + *CDK2AP1* for each gene analyzed). Results are presented together with standard deviation from experiments conducted in triplicate.



**Figure 29: Simultaneous Knockdown of CDK2AP1 and p53 Prevents the G2/M Accumulation and the Decrease in S phase Seen in the CDK2AP1 only Knockdown hESCs.** H9 hESCs were co-transduced with CDK2AP1 and p53 shRNAs, stained with propidium iodide and analyzed by flow cytometry to examine their cell cycle profile. Knockdown of CDK2AP1 in H9 hESCs resulted in a decrease in the percentage of cells in the S phase and increased cells in the G2/M phase of the cell cycle (Figure 22). When p53 is simultaneously downregulated with CDK2AP1, there was no reduction in cells in the S phase and no increase in cells in the G2/M phase of the cell cycle. Around 6% of the analyzed cells were in the sub G0/G1 phase of the cell cycle. Results are presented together with standard deviation from experiments conducted in triplicate. Cell cycle data of EV and CDK2AP1-shRNA transduced hESCs from figure 22 were included in this figure to facilitate comparison.

To investigate the function of CDK2AP1 in human pluripotent stem cell self-renewal, pluripotency and differentiation potential, we knocked down CDK2AP1 expression in multiple human pluripotent stem cells by RNA interference. Using a lentiviral approach, we were able to downregulate CDK2AP1 by 97%. When we examined the levels of *OCT4* and *NANOG* in the CDK2AP1 knockdown hESCs, we found that the knockdown resulted in a decrease in the transcription of these genes (CDK2AP1-shRNA1 resulted in a 60% reduction in *OCT4* and *NANOG* and when CDK2AP1-shRNA2 was used, knockdown resulted in a 70% reduction in *OCT4* and 50% reduction in *NANOG* expression). These results indicated a possible difference in the role of CDK2AP1 in hESCs when compared to mESC, in which when *Cdk2ap1* was deleted, *Oct4* expression was sustained (Deshpande, Dai et al. 2009).

Our results also indicated that CDK2AP1 knockdown hESCs had a greater propensity to differentiate when EB formation was induced. When we examined the expression of markers of the germ layers in 18-21 day old EBs, we found that EBs generated from CDK2AP1 knockdown hESCs exhibited higher expression of candidate markers of all the three germ layers. The difference was especially evident in the expression of mesoderm (27.6 fold increase in *T*) and in the ectoderm markers (67.2 fold increase in *SOX1*). Histopathological analysis also revealed that EBs generated from CDK2AP1 knockdown hESCs primarily demonstrated mesoderm and ectoderm differentiation, while EBs generated from wild type hESCs routinely demonstrated exclusive differentiation towards neuroectoderm. Taken together, our results indicate that knockdown of CDK2AP1 in hESCs enhances differentiation by yielding more EBs that have significantly higher expression of markers of all three germ layers.

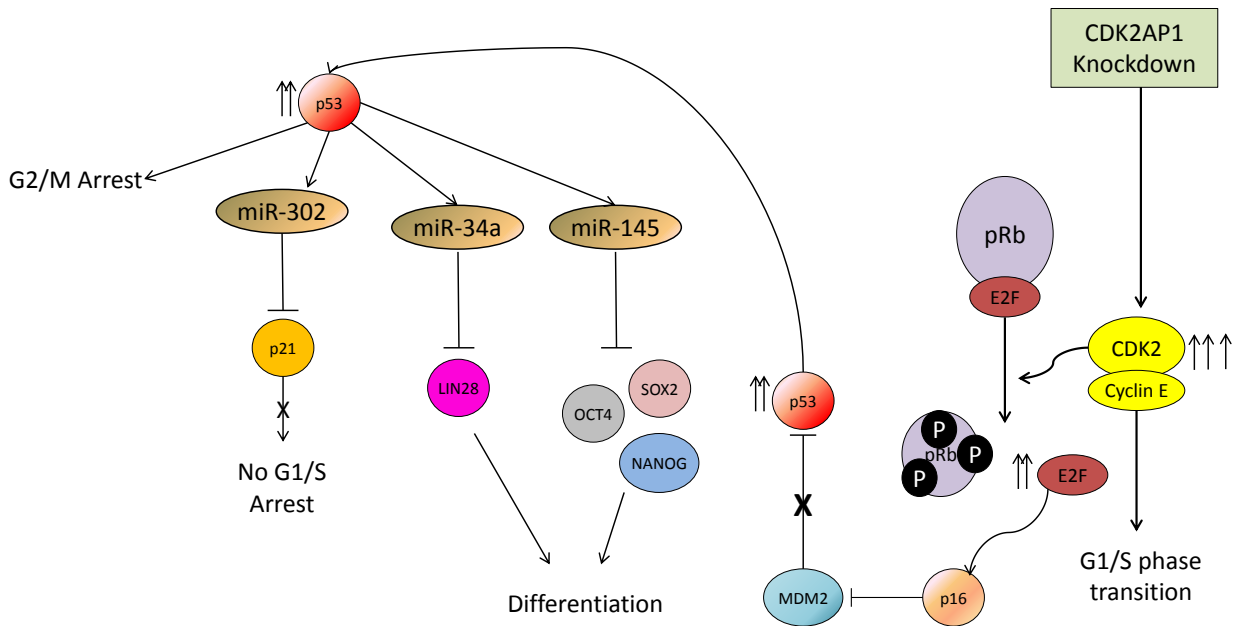
Given the close connection between cell cycle and pluripotency, we next aimed to examine the effect of CDK2AP1 knockdown on the proliferation and the cell cycle of hESCs. We initially observed that CDK2AP1 knockdown hESCs were proliferating significantly slower and had to be sub-cultured less frequently. When the cell cycle profile of these cells was examined by propidium iodide staining and flow cytometry analysis, we found that these cells accumulated in the G2/M phase of the cell cycle and had a significantly lower percentage of cells in the S phase of the cell cycle. We also found that a higher percentage of these cells stained positive for p-H3, which is detected in cells that are in the late G2 and M phase of the cell cycle. In addition, Cyclin A1 and p53 protein levels were increased following CDK2AP1 knockdown by 2 and 1.76 folds respectively. It has been reported previously, that increased p53 levels can increase Cyclin A1 which in turn can cause G2/M arrest (Rivera, A. et al. 2006).

Increased p53 levels can also cause arrest in the G2/M phase of the cell cycle in a number of ways that are independent of Cyclin A1. It is known that when levels of p53 are increased, it results in the induction of the expression of the 14-3-3 $\sigma$ , a gene originally discovered through its expression in differentiating epithelial cells and a member of the 14-3-3 protein family (Prasad, Valverius et al. 1992, Hermeking, Lengauer et al. 1997). Through a p53-responsive element located 1.8 kb upstream of its transcription start site, 14-3-3 $\sigma$  is induced upon p53 binding and inhibits G2/M progression by sequestering CDK1 in the cytoplasm (Hermeking, Lengauer et al. 1997). In addition, p53 can exert its inhibition of the G2/M transition by decreasing intracellular levels of cyclin B1 protein and attenuating the activity of the cyclin B1 promoter (Innocente, Abrahamson et al. 1999). Furthermore, p53 has also been shown to repress the promoter of CDK1, which is another way through which G2/M transition is regulated by p53 (Passalaris, Benanti et al. 1999).

We observed that when p53 and CDK2AP1 were both downregulated, there was minimal reduction in *OCT4* and *NANOG* expression, suggesting the observed reduction in their levels following CDK2AP1 may be p53 dependent. A recent study showed that upon the induction of differentiation of hESCs, there was an enrichment in p53 binding to p53 responsive elements in the promoters of miR-145 (Jain, Allton et al. 2012), which then suppresses the expression of the pluripotency genes, *OCT4*, *SOX2* and *KLF4* in hESCs and promotes differentiation (Xu, Papagiannakopoulos et al. 2009). In addition, when the cell cycle profile of H9 hESCs that were co-transduced with CDK2AP1 and p53 shRNAs was examined, we found that the introduction of the p53 shRNA prevented the decrease in S phase and the increase in the G2/M phase of the cell cycle that was seen in the CDK2AP1 knockdown only hESCs. This data suggests that the change in the cell cycle profile following CDK2AP1 knockdown may be caused due to an increase in p53 levels. Being an inhibitor of CDK2 we expect that the knockdown of CDK2AP1 might have temporarily alleviated this inhibition leading to faster progression into S phase and an aberrant upregulation of the oncogene E2F. This in turn may cause a p16 (ARF) mediated degradation of the p53 inhibitor MDM2 leading to an increase in p53 levels (figure: 30) (Zhang, Xiong et al. 1998).

Although CDK2AP1 knockdown in hESCs resulted in a decrease in *OCT4* and *NANOG* levels, these cells differentiated better than wild types. The knockdown of CDK2AP1 improved the generation of EBs and enhanced the expression of all tested markers of the three germ layers. Overall, our results indicate that hESCs exhibit reduced self-renewal potential following CDK2AP1 knockdown, a significantly lower percentage of cells in the S phase, and higher percentage were in the G2/M phase when compared to wild type cells. Therefore, our results lead us to the conclusion that the knockdown of CDK2AP1 in hESCs lowers the threshold of

differentiation by decreasing self-renewal and increasing the susceptibility of these cells to differentiate.



**Figure 30: The Expected Model Following CDK2AP1 Knockdown in hESCs.** CDK2AP1 knockdown may result in an increase in CDK2 activity leading to upregulation of the oncogene E2F. This will in turn increase p53 levels. Increased p53 levels in hESCs has been reported to result in G2/M arrest and to increase the expression of the miRNAs 302, 34a and 145. MiR-302 is known to downregulate p21, thus preventing the G1 arrest in hESCs. MiRNA's 34a and 145 downregulate the pluripotency genes, LIN28, OCT4, NANOG and SOX2.



## **CHAPTER 4**

### **Conclusions and Future Directions**

#### **Conclusions:**

CDK2AP1 has been shown to mainly function as a cell cycle regulator that inhibits CDK2 by targeting it for proteolysis, therefore inhibiting the progression from G1 to S phase. It has also been reported that CDK2AP1 binds the DNA polymerase/alpha primase complex and inhibits the initiation step of DNA synthesis. CDK2AP1 overexpression in a number of cancer cell lines decreased invasion and reduced proliferation. In the first study we aimed to investigate the effect of CDK2AP1 downregulation on the growth of normal primary human fibroblasts. The results obtained from the experiments support the following conclusions:

- Knockdown of CDK2AP1 in primary HDFs reduces proliferation

- CDK2AP1 knockdown causes an accumulation of cells in the G1 phase of the cell cycle
- Knockdown of CDK2AP1 in primary HDFs induced premature senescence
- When compared to wild type HDFs, CDK2AP1 knockdown HDFs have higher  $\gamma$ -H2AX signal, representative of DNA damage
- Knockdown of CDK2AP1 increases mRNA and protein levels of p53
- Knockdown of CDK2AP1 increases mRNA levels of *BAX*, *PUMA* and *p21*
- The observed senescence and increase in G1 phase of the cell cycle following CDK2AP1 knockdown is p53 dependent

In the second study, we investigated the effect of CDK2AP1 knockdown on the self-renewal and pluripotency of hESCs. Recent reports showed that CDK2AP1 plays an important role during the differentiation of mESCs. *Cdk2ap1* null mESCs fail to differentiate properly, and experienced persistent self-renewal, evident by increased proliferation and hyperphosphorylation of the retinoblastoma protein. It was also shown that CDK2AP1 is required for silencing of *Oct4* expression during mESC differentiation. Based on these findings, it seems that deletion of *Cdk2ap1* in mESCs favors self-renewal over differentiation. To investigate the effect of CDK2AP1 downregulation on the self-renewal and pluripotency of hESC, we used lentiviral delivery of CDK2AP1 specific shRNA to create stable CDK2AP1 knockdown hESCs cell lines.

Series of experiments conducted with the CDK2AP1 knockdown hESCs led to results which supports the following conclusions:

- CDK2AP1 knockdown lowers the differentiation threshold of hESCs. This was demonstrated by the reduced levels of OCT4 and NANOG and by the enhanced ability of these cells to differentiate and form EBs.
- CDK2AP1 knockdown increases the expression of all tested markers of the three germ layers in the generated EBs (Endoderm: *AFP* and *GATA4*. Mesoderm: *T* and *IGF2*. Ectoderm: *SOX1* and *NESTIN*).
- The knockdown of CDK2AP1 significantly slowed cell division in hESCs and reduced the percentage of cells in the S phase and significantly increased the percentage of cells in the G2/M phase of the cell cycle.
- CDK2AP1 knockdown led to increase in Cyclin A levels.
- CDK2AP1 knockdown increased p53 protein levels
- Simultaneous knockdown of CDK2AP1 and p53 prevents the reduction in *OCT4* and *NANOG* levels
- Simultaneous knockdown of CDK2AP1 and p53 prevents the reduction of cells in the S phase and the increase in the G2/M phase of the cell cycle.

## **Future Directions**

Human pluripotent stem cells that include hESCs and hiPSCs represent a good alternative to adult stem cells as a source of an unlimited supply of well-defined pluripotent stem cells that can generate clinical-grade, transplantable, lineage-specific cells. Our data demonstrated enhanced differentiation potential when CDK2AP1 is downregulated in hESCs. Higher numbers of EBs were obtained in which levels of markers of all three germ layers were significantly higher. Neuroectoderm markers, *Sox1* and *Nestin*, expression was increased 67 and 4 folds respectively in CDK2AP1 knockdown EBs when compared to wild type EBs. We therefore propose the investigation of the effect of CDK2AP1 knockdown on the differentiation of hPSCs to neural progenitor cells. We expect that due to increased expression of these markers in the knockdown EBs, generation of neural progenitors would be enhanced. This study could be accomplished through the following specific aims:

- Specific Aim 1: Differentiate both CDK2AP1 WT and knockdown hPSCs to neural progenitors.

Using established differentiation protocols both wild type and knockdown cells would be directed to differentiate to form neural progenitors. The purpose of this aim is to assess the ability of CDK2AP1 knockdown hPSCs to differentiate efficiently to neural progenitors. The quantity and the quality of the generated neural progenitors will be investigated. The generated cells will be examined for the formation of neural rosettes, a characteristic of neural progenitors. The purity of the generated neural progenitors will be examined by flow cytometry by measuring the levels of candidate neural progenitor markers (NESTIN and MUSASHI1). In addition, qPCR analysis will be conducted to compare the marker expression between wild type and knockdown hPSCs.

- Specific Aim 2: Examine the ability of generated neural progenitors to efficiently form multiple cell types of the nervous system.

In experiments conducted under this aim, CDK2AP1 knockdown and WT neural progenitors will be differentiated in culture to form neurons, oligodendrocytes and astrocytes. Gene expression analysis, immunocytochemistry, and FACS analysis will be utilized to compare expression of multiple markers specific to the different differentiated cell types. In addition, the potential of wildtype and the CDK2AP1 knockdown neural progenitors to form specialized neuronal cells like motor neurons and dopaminergic neurons for clinical applications will be compared.

Previous studies have focused on investigating the potential of overexpressing CDK2AP1 in cancer cell lines and determine its effect of cell growth and apoptosis. However, few studies have investigated the effect of CDK2AP1 knockdown on cancer cell lines. In studies conducted with primary human dermal fibroblast, we found that the knockdown of CDK2AP1 induced premature senescence, reduced proliferation and increased the expression of *p53*, *p21* and the p53-responsive apoptotic genes, *BAX* and *PUMA*. We thus propose to investigate the effect of CDK2AP1 downregulation on the proliferation characteristics of a primary cancer cell line. This study will be accomplished through the following specific aims:

- Specific Aim 1: Examine the effect of CDK2AP1 knockdown on a human primary cancer cell line.

A primary cancer cell line (e.g. breast cancer cell line) will be transduced with a scrambled sequence shRNA or an shRNA directed against CDK2AP1. After confirming successful

knockdown, the effect of the knockdown on cell proliferation will be examined by measuring doubling time, MTT assay or BrdU incorporation assay. In addition, the cell cycle profile of knockdown and wild type cells will be compared. Next, the effect of CDK2AP1 knockdown on the levels of cell cycle regulators like, p53, p21, p16, BAX and PUMA will be examined by gene expression and Western analysis. Cell invasion assay will also be performed to determine the effect of the knockdown on the invasiveness of the cancer cells.

- Specific Aim 2: Utilize an in vivo model to examine the effect of CDK2AP1 knockdown on tumor formation.

CDK2AP1 wild type and knockdown cells will be subcutaneously injected into nude mice and tumor growth monitored. We have observed decreased BrdU incorporation when CDK2AP1 was downregulated in primary fibroblasts and increased expression of the apoptotic genes *BAX* and *PUMA*. We will thus conduct experiments to monitor proliferation and apoptosis in the CDK2AP1 knockdown tumor cells by performing BrdU and TUNEL assay on tumor tissue.

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## **APPENDIX**

### **Bromodeoxyuridine (BrdU) Proliferation Assay**

1. Seed cells to be assayed in the appropriate density in 4-chamber slides and incubated in 37 degrees, 5% CO<sub>2</sub> incubator overnight.
2. The next day, BrdU (Sigma, Cat# B9285-250G) is added to the medium in 20 µg/ml final concentration. Put the slides back in the incubator for 4-6 hrs.
3. After 4-6 hrs, cells are washed with ice cold PBS buffer and then fixed in 100% methanol in -20C for 15 min.
4. After fixing, the cells were washed in cold PBS and permeabilized by adding 0.2% Triton-X for 15 min at room temperature.
5. For DNA denaturation (antigen retrieval), cells are treated with 2N HCL solution for 45 min at room temperature.
6. To neutralize the HCL, Borate buffer is added for 6 min and washed. This should be done twice to insure neutralization of HCL.
7. After that blocking using 3% goat serum is performed for 45 min at room temperature.

8. Remove blocking and add primary BrdU antibody (Abcam, Cat# 1893) at (1:500 dilution) and incubate overnight at 4C.
9. The next day, wash cells three times with PBS buffer with 5 min incubation for every wash.
10. Secondary antibody (Goat anti-Rat, Alexa fluor 594, Cat# A-11007) is then added and incubated at room temperature for 1 hr.
11. Cells were then washed with PBS and labeled with DAPI (1:10,00 dilution in deionized water)
12. To calculate the percentage of BrdU positive cells, view slides at 20X magnification and Select random fields. Determine the total number of cells in each field by counting nuclei (DAPI). Determine the number of BrdU positive cells in the field. Calculate the percentage by dividing the number of BrdU positive cells by the total number of cells in the field x 100.

### **Immunoblotting (Western Blot)**

1. Sample preparation
  - Cells must be confluent and ready to be harvested from a 60mm cell culture plate.
  - Use the total protein extraction kit which has 50x Protease inhibitor cocktail + TM lysis buffer (with NP40). Make 1x Protease inhibitor by diluting it from 50x using lysis buffer.

- Wash the cell plate with PBS after aspirating the media out. Be careful not to leave any traces of media in the plate as this will obstruct viewing the protein of interest on SDS PAGE later on.
- Use ice cold PBS and cold tubes for the rest of the sample preparation.
- Trypsinize the cells and spin them down.
- Wash the cell pellet with PBS.
- Add 100 µl of lysis buffer to 1 million cells.
- Pipette it up and down.
- Tape the tubes down to a vortex and keep it on constant agitation at 4°C for 30 minutes.
- Centrifuge at 13000 g at 4°C for 15-20 minutes.
- Collect the supernatant and dump the pellet. The supernatant is the protein.

## 2. Protein concentration: BSA assay

- Dye reagent (5x)
- BSA lyophilized powder
- Add 20 ml water to do the BSA powder and mix it to get a 1.44 mg/ml protein solution.
- Make three dilutions of protein solution (0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml).

0.8 mg/ml: 1.44 mg – 1ml

$$0.8 \text{ mg} - 0.8/1.44 = 0.556 \text{ ml}$$

Add 0.444 ml dd H<sub>2</sub>O

1ml of 0.8 mg/ml solution

0.4 mg/ml: Take 0.5 ml of 0.8 mg/ml and add 0.5 ml of dd H<sub>2</sub>O to get 1 ml of 0.4 mg/ml solution.

0.2 mg/ml: Make 1ml of protein solution in the same way as above.

- Dye reagent: 1 part of 5x dye + 4 parts water = 25 ml dye (1x)  
Pour the dye reagent through a Whatman filter paper.
- Take 20 µl of protein (standards/sample) for 1ml of dye reagent and vortex it properly and incubate it for 5min at room temperature.
- Perform spectrophotometric analysis at 595nm to get the OD values.
- Only 30 µg of protein per well in gel. Do the calculations and take the required amount of protein sample and make up the volume to 30 µl using laemmli buffer (1:1 or 1:2).
- Before using the laemmli buffer, it is required to add β-mercaptoethanol to the laemmli buffer.
- Add 50 µl β-mercaptoethanol to 950 µl Laemmli buffer and mix it well.
- Add 1:1 ratio of Laemmli buffer to protein sample.
- Boil water on a burner, parafilm-seal the lids of the eppendorf tubes that contain sample and place the samples in boiling water for around 10 minutes.
- Spin them down to the bottom of the tube.

### 3. Running SDS PAGE

- Making running buffer: Take 900ml ddH<sub>2</sub>O in a glass bottle and add 100ml of Tris-Glycine-SDS and mix it well.
- Once the samples and buffers are ready, take them near the running apparatus.
- Get the gel ready from 4°C and take the tape off the bottom of the gel.
- Place it carefully in the gel holder and make sure to place the gel in a way that the wells are facing inside. Balance the other side using another gel if you are running two gels at the same time or use a dam to seal the other end.
- Pour running buffer into the gel holder to check if it is sealed well.
- Once it is established that there are no leaks, go ahead and take the comb out of the wells and saturate it with running buffer.
- Load ladder and samples carefully into the respective wells without releasing air bubbles into the well from the pipette.
- Run at 20mA for around 45min or till the protein reaches the bottom of the gel. Be careful not to let the protein run out from the bottom of the gel. If the gel has run well, the ladder is well separated.

#### 4. Transfer onto nitrocellulose membrane

- Preparation: While the SDS PAGE is still running, get the transfer buffer ready and store it at 4°C.
  - Take 700 ml water, 100 ml tris-glycine, 200 ml methanol and mix it well.
  - Take ice in white ice-holder and fill it with a little water and keep it at - 20°C until use.
  - Cut the required size of the nitrocellulose and be careful not to touch without gloves and hold it using forceps. Place it in some transfer buffer.

- Wet the sponges and filter paper in the transfer buffer as well.
- Once the gel is run, carefully break open the gel case without breaking the gel.
- Place the nitrocellulose on gel supported by a filter paper.
- Let the gel come onto the membrane and then place another filter paper on gel.
- Press them smoothly and now place sponges on either side and roll a Pasteur pipette on this sandwich tightly to get rid of bubbles. (IMPORTANT)
- Do not forget the gel side and the membrane side.
- Black side: Gel  
Clear side: Nitrocellulose membrane
- Clear side should go next to red and black side onto black and back goes back.  
Place the other sandwich with another set of gel-membrane or plain sponges.
- Place the ice pack in the apparatus and place the whole apparatus in a trough containing ice.
- Now pour the cold transfer buffer into the apparatus and run it at 90V for around 1 hour 15 min-30min.
- You know the transfer has begun once you notice bubbles rising up from the bottom.
- The transfer is successful when the ladder is perfectly visible on the nitrocellulose membrane.

##### 5. Blocking and washes:

- Use 3% block solution or make 5% block using nonfat casein powder in TBS-Tween solution
- TBS-Tween: 900ml water, 100ml TBS, 1ml Tween-20
- Do a 10 min wash with TBS-Tween.

#### 6. Primary and Secondary Antibodies:

- Make the primary antibody solution in 0.1% TBS-Tween solution (15ml). It can be reused when stored at 4°C.
- Add it to the washed membrane and leave it overnight at 4°C with constant agitation or upto 2 hrs at room temperature with stirring.
- Do 3x 10 min washes with TBS-Tween.
- Make secondary antibody in TBS-Tween (10ml).
- Add it onto membrane and leave it on stirring for 1 hour.
- Do not reuse the secondary antibody solution.

Do 3x 10 min washes with TBS-Tween and detect using the Odyssey Infrared Imaging System (Li-Cor Biosciences, NE).

#### **CDK2AP1 knockdown**

CDK2AP1 specific shRNAs were purchased from OpenBiosystems, PA and SIGMA, MO.

ShRNAs were received in glycerol stocks.

- Use a sterile pipette tip to inoculate a small amount of the glycerol stock of the shRNA and streak on an agar plate that has the appropriate antibiotic.
- Place the agar plates upside down in an incubator at 37 C overnight.

- The following day, observe the plates for resistant colonies.
- Using a sterile pipette tip pick well isolated colonies and insert it in a glass tube containing 3-5 mls of LB medium with appropriate antibiotic.
- Place the tubes in the 37 incubator/shaker for 16-18 hrs in constant agitation at 280 rpm.
- Dilute 0.5-2 mls of the liquid bacterial culture prepared in the previous step in 100 mls LB medium with the appropriate antibiotic and incubate at 37 C at constant 280 rpm agitate overnight.
- The next day, collect liquid culture and extract plasmid using the Qiagen Maxi Prep kit (Cat number 12963) by following the manufacturer's directions.
- Using the spectrophotometer, determine the plasmid concentration.
- To generate lentiviruses containing shRNA:
  - Seed HEK293 cells onto 10 cm cell culture plate.
  - When cells reach ~ 70% confluency, transfect with 10 µg of the shRNA plasmid + 7.5 µg packaging plasmid (psPAX2) (Addgene, MA) + 2.5 µg envelope plasmid (pMD.2) (Addgene, MA). Use Roche transfection reagent, X-tremeGENE.9.
  - 48 h later, collect supernatant which contains the viral particles and filter it using a 0.45 µm filters.
  - Concentrate the viral supernatant using the Lenti-X concentrator (Clontech, CA) by following the manufacture's protocol.
  - Resuspend the viral particles in 500 µl Opti-MEM and aliquot in 50 µl and store the tubes at -80 C for later use.
- To knockdown CDK2AP1, seed around 100k cells per 35 mm dish (this density is optimized for human embryonic stem cells (hESCs) and primary human fibroblasts



(HDFs). Adjustments may need to be made depending on the cell line you are transducing).

- Add ~25  $\mu$ l of virus per 35 mm plate with the addition of Polybrene (for hESCs and HDFs use 6  $\mu$ g/ml) (Santa Cruz Biotech, CA).
- The next day, remove transduction medium and add growth medium.
- When cell become confluent, passage the cells 1:2 or 1:3 to increase the number of transduced cells.
- The next day, add Puromycin to select the successfully transduced cells. (use 3  $\mu$ g/ml for HDFs and 0.5-1  $\mu$ g/ml for hESCs).
- Resistant cells can be collected following ~5 days of antibiotic treatment.
- Determine the degree of knockdown using qPCR or Western Blot.

### **Cell Cycle Analysis by Propidium Iodide (PI) Staining**

- To prepare PI staining solution stock:
  - Add 100 ml of Tris Buffered Saline (pH = 7.6)
  - Add 1 mg of Ribonuclease A (Type-II; 70 U/mg) (Sigma #R-5000)
  - Weigh 7.5 mg of Propidium Iodide (Sigma #P-4170) and add it to the solution
  - Add 0.1 ml of IGEPAL CA-630 (Sigma #I-8896).
  - Cover the container with foil and store at 4 C
- Place 500k -10<sup>6</sup> cells in 12 x 75 mm tube
- Centrifuge the cells at 250g for 5 minutes.
- Discard supernatant and resuspend cells in 0.5-1 ml of the prepared PI solution.
- Incubate at 37 C for 45 min

- Before analysis, Samples need to be filtered through a 30µm mesh to eliminate clumps.
- Analyze on Accuri flow cytometer at low flow rate (~150 cells/second or less).
- Threshold debris using Forward Scatter, then gate using PI-area vs. PI-height to eliminate doublets. Viable nuclei will have some Forward Scatter and little 90 degree –Scatter.
- Analyze cells using PI-area.

## **Vita**

Khaled N. Alsayegh was born in Tunis, Tunisia on January 12, 1983. He attended and graduated from high school in Jeddah, Saudi Arabia in 2000. He then began his undergraduate studies at King Abdul Aziz University in Jeddah, in Medical Technology. He received his bachelor degree in the year of 2005. Khaled received King Abdullah scholarship to pursue his graduate studies in the United States in 2006. In 2009, he received his Master's degree in Human and Molecular Genetics at Virginia Commonwealth University. Since 2009, Khaled has been pursuing his Doctorate degree in Human and Molecular Genetics. His future plan is to return to his home country and work in academia at one of the Universities there.